PASTEURELLA HAEMOLYTICA LEUKOTOXIN AND ITS INTERACTION WITH TARGET CELLS

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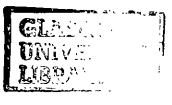

DECLARATION

The thesis is the original work of the author:

M. Saadati

DEDICATION

This thesis is dedicated to my parents, who supported me tirelessly throughout my academic career.

It is also dedicated to my wife, Zohre, and my children, Hamideh and Mohammad hossien, who are always sources of mental support, happiness, love and inspiration for my future.

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ABBREVIATIONS

A1	*P. haemolytica* biotype A capsular serotype 1
A2	*P. haemolytica* biotype A capsular serotype 2
A5	*P. haemolytica* biotype A capsular serotype 5
A6	*P. haemolytica* biotype A capsular serotype 6
A7	*P. haemolytica* biotype A capsular serotype 7
A8	*P. haemolytica* biotype A capsular serotype 8
A9	*P. haemolytica* biotype A capsular serotype 9
A11	*P. haemolytica* biotype A capsular serotype 11
A12	*P. haemolytica* biotype A capsular serotype 12
A13	*P. haemolytica* biotype A capsular serotype 13
A14	*P. haemolytica* biotype A capsular serotype 14
A16	*P. haemolytica* biotype A capsular serotype 16
A17	*P. haemolytica* biotype A capsular serotype 17
Ab	Antibody
Abs	Absorbance
AC	Adenylate cyclase
Ag	Antigen
Ap	Ampicillin
API	Analytical profile index
Approx.	Approximate
ApxIA	*Actinobacillus pleuropneumoniae* RTX-toxin IA
ApxIIA	*Actinobacillus pleuropneumoniae* RTX-toxin IIA
ApxIIIA	*Actinobacillus pleuropneumoniae* RTX-toxinIIIA
ATP	Adenosine triphosphate
Av.	Average
BHIA	Brain heart infusion agar

BHIB	Brain heart infusion broth
BN	Bovine nasopharynx
BP	Bovine pneumonic
BSA	Bovine serum albumin
C.	Circa (approx)
°C	Degrees Celsius
CL	Chemiluminescence
cm	centimetre
Cm	Chloramphenicol
Conc.	Concentration
CPS	Capsular polysaccharide
cya	adenylate cyclase gene
Cya	adenylate cyclase phenotype
DAB	3-3'-diaminobenzidene tetrahydrochloride
Dist. water	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNDH	Dimethylamino-naphthalene-1,2-dicarbonic acid hydrazide
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis N,N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
fMLP	Formyl methionine-leucine-phenylalanine
g	Gram (s)
h	Hour (s)

H_2O	Distilled water
HA	Haemaglutination
Hb	Haemoglobin
HEPES	(N-2-hydroxyethylpiperazine-N'-2-ethanosulphonic acid)
HH	Hanks Hepes
Hly	Haemolysin
hly	Haemolysin gene
HlyA	Haemolysin protein
HMPS	Hexose monophosphate shunt
HRP	Horseradish peroxidase
i/m	Intramuscular
Ig	Immunoglubulin
IHA	Indirect haemagglutination
IPTG	Isopropyl-ß-D-thiogalactopyranoside
IRP	Iron-regulated protein (s)
kbp	Kilobase pairs
kDa	Kilo Dalton
l	Litre
lac	Lactose operon gene
LB	Luria-Bertani
LDCL	Luminol-dependent chemiluminescence
lkt	leukotoxin gene
Lkt	Leukotoxin
LktA	Leukotoxin protein
LPS	Lipopolysaccharide
µg	Microgram (s)
µl	Microlitre (s)

µm	Micrometre (s)
M	Molar
mA	Milliampere (s)
Mab	Monoclonal antibody
mg	Milligram (s)
min	Minute (s)
ml	Millilitre (s)
mm	Millimetre (s)
mM	Millimolar
Mol. wt	Molecular Weight
NA	Nutrient agar
NB	Nutrient broth
NCIMB	The National Collection of Industrial and Marine Bacteria
NCTC	The National Collection of Type Cultures
OD	Optical density
OM	Outer membrane
OMP	Outer-membrane protein (s)
OP	Ovine pneumonic
OPD	O-phenylene diamine
OZ	Opsonised zymosan
Pab	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
pH	Hydrogen ion concentration
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear
psi	Pounds per square inch

R	Rough (lipopolysaccharide)
RBC	red blood cell
rcf	Relative centrifugal force (x g)
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTX	Repeats in toxin
S	Smooth (lipopolysaccharide)
s/c	Subcutaneously
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Second (s)
T3	*P. haemolytica* biotype T capsular serotype 3
T4	*P. haemolytica* biotype T capsular serotype 4
T10	*P. haemolytica* biotype T capsular serotype 10
T15	*P. haemolytica* biotype T capsular serotype 15
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris hydrochloride
TSB	Transformation and storage buffer
UT	Untypable
V	Volt (s)
v/v	Volume/volume ratio
w/v	Weight/volume ratio

SUMMARY

A number of *Pasteurella haemolytica* isolates from cattle and sheep, including representatives of all serotypes and untypables, was examined for leukotoxin (LktA) production at the end of the log phase of growth in brain-heart infusion broth. The bacteria produced most toxin with very high and high aeration, at 37 °C, in a low concentration of NaCl and at alkaline pH (pH 8). There were marked differences in leukotoxic activity in culture supernate samples, as measured by chemiluminescence-inhibition assays with bovine and ovine neutrophils, even between strains of the same serotype. There was also some variation in the amount and mol. wt of the LktA protein produced by different strains, as judged by SDS-PAGE, immunoblotting and ELISA. Some strains produced normal amounts of LktA protein which had only low leukotoxic activity. Most strains (28/32) produced LktA of 105 kDa whereas four strains produced a higher mol. wt form of c.108 kDa, including two of the five serotype A2 strains examined. Thus, the *P. haemolytica* isolates showed considerable heterogeneity in terms of leukotoxin production, mol. wt and activity, even within a given serotype. Serial passage of *P. haemolytica* did not affect the production of leukotoxin (LktA). The mol. wt of LktA was more than 300 kDa as judged by ultrafiltration, due presumably to aggregation. Attempts to disaggregate LktA with SDS, zwittergent, EGTA and urea were not successful.

Although *P. haemolytica* can produce LktA in the absence of calcium, for activity of LktA, calcium was found to be necessary. Active and non-activated rLktA was prepared after transformation of *lktA* and *lktC* genes into different strains of *E. coli.* The biological activities of active recombinant LktA were the same as that of native LktA, but the amount of leukotoxin obtained from *E. coli* was greater than that from *P. haemolytica.*

Although previous reports had shown that LktA affected and killed only ruminant leukocytes, in this study it was found that LktA had different effects on ruminant and non-ruminant neutrophils. Leukotoxin killed ruminant neutrophils and bovine lymphoma (BL3) cells by cell swelling and lysis, but it had no effect on rabbit and guinea pig neutrophils and a mouse macrophage cell line (J774.2). Surprisingly, native and recombinant LktA partially inhibited the CL response of human neutrophils, although it did not kill the cells. This partial inhibition was due to leukotoxin and not to other components of bacteria such as LPS. Slightly different results were found by a cell tracking assay in which the neutrophils were incubated with active and non-activated recombinant LktA. The active toxin killed bovine neutrophils, but caused the migration of human neutrophils. However, the same concentration of non-activated rLktA had no effect either on CL response or movement of human neutrophils. The reason why LktA promoted movement of human neutrophils, but partially inhibited the CL response deserves further investigation as it suggests two opposing effects of LktA i.e. inhibition of chemiluminescence and production of cell movement.

Although non-activated leukotoxin had no effect on CL response of different ruminant and non-ruminant target cells, it bound to these cells in a similar way to that of the active form of rLktA. The active and non-active LktA bound to target cells at 4 °C, but to a lesser extent than at 37 °C but no lysis of ruminant cells was observed at 4 °C, indicating that binding and subsequent lysis can be dissociated and probably represent two separate events.

Different monoclonal antibodies were used in an attempt to neutralise the toxicity of LktA, but none of them could completely neutralise its activity. A polyclonal antibody raised against rLktA completely neutralised the homologous LktA but only partially neutralised heterologous LktA from other strains of *P.*

haemolytica. In contrast, bovine convalescent serum neutralised homologous and heterologous LktA.

In addition to its leukotoxin activity, LktA also has haemolytic activity. This activity is weak and variable in different isolates of *P. haemolytica*. Non-activated LktA had no haemolytic activity.

Non-activated rLktA was activated *in vitro* in the presence of LktC and cytosolic activating factor (CAF). This activated toxin affect bovine neutrophils and had some effect on rabbit neutrophils, as judged by CL inhibition assay. However, lower LktA activity was found with the *in-vitro*-activated toxin than with *in-vivo*-activated rLktA.

1. INTRODUCTION

1.1 PASTEURELLA HAEMOLYTICA-THE ORGANISM

1.1.1 History

According to the present classification, the 'Family *Pasteurellaceae*' consists of three genera of Gram-negative, facultatively anaerobic and fermentative bacteria, namely: *Actinobacillus, Pasteurella* and *Haemophilus* (Mannheim, 1984). The name of the genus *Pasteurella* was proposed by Count Trevisan in 1887 (cited by Carter, 1984) in recognition of the work of Louis Pasteur, the early investigator of fowl cholera epidemics in turkeys. The early classification of members of the genus was based on the place of isolation, the type of lesion found in the particular animal or the person who worked with them. Currently, six species are recognised; *P. multocida, P. pneumotropica, P. ureae, P. gallinarum, P. aerogenes and P. haemolytica* (Carter, 1984; Mutters *et al.*, 1986). These species are differentiated by their ability to produce beta haemolysis, growth on MacConkey's agar, indole production, urease activity, gas from carbohydrates and acid production from lactose or mannitol.

Pasteurella strains have also been examined by other techniques such as DNA/DNA hybridization, 2-dimensional protein electrophoresis and rRNA sequence analysis which have resulted in a proposal for a new classification of *Pasteurella* into 11 species in the genus *Pasteurella sensu stricto* (Confer, 1993). With these methods, a number of subspecies of *P. multocida* are recognised and these tend to be relatively animal-species specific. In fact, rRNA analysis has suggested that *P. haemolytica* may not even belong in the genus of *Pasteurella sensu stricto* (Dewhirst *et al.*, 1992).

Pasteurella species are Gram-negative, ovoid or rod shaped 0.25-0.4 μm in width and 0.6-2.5 μm in length, non-motile, catalase-positive, facultative

anaerobic, gelatinase-negative and commonly show bipolar staining (Carter, 1984).

P. multocida is an important pathogen of animals and man. Although found in the nasopharynx of clinically healthy animals as a part of the normal nasal flora, it can cause a number of diseases such as fowl cholera in birds and haemorrhagic septicaemia in cattle. Apart from having an endotoxin (LPS) similar to that of other Gram-negative bacteria, some strains produce a protein toxin that is lethal for mice, guinea pigs, swine and turkeys. This species is often pleomorphic, with bipolar staining, but is usually in the coccobacillary and rod form (Carter, 1984).

P. pneumotropica is a commensal in the upper respiratory tract of laboratory animals including mice, rats and guinea pigs and produces disease in ducklings as well as turkeys. This bacterium grows on blood agar but does not produce haemolysis (Carter, 1984).

P. ureae is infrequently found in the respiratory tract of healthy humans and it can occasionally produce disease (Mannheim, 1984). Medium with blood or serum is best for growth of this bacterium (Carter, 1984).

P. gallinarum cannot grow on MacConkey's agar and does not produce haemolysis on blood agar. This species is isolated from chickens and turkeys (Hall *et al.*, 1955).

P. aerogenes can grow on MacConkey's agar but it cannot grow on Salmonella-Shigella agar. It is non-capsulated and does not produce visible growth at 25-28 °C in 24 hours. The organism occurs in domestic swine and wild boars and is only seldom isolated from man (Frederiksen, 1989).

Another organism, *P. anatipestifer* occurs worldwide in both domestic and wild birds. Although strain P-2361 (serotype 7) of *P. anatipestifer* produces haemolysis in blood agar, haemolysis is not a normal characteristic of this species (Brogden *et al.*, 1989). This is a non-fermentative organism and

does not properly belong in the genus of *Pasteurella*, according to Carter (1984).

The first observations on *P. haemolytica* were reported by Jones (1921) after studying bovine bacterial isolates. He divided these isolates into three groups and located atypical strains in group one. This group fermented lactose, maltose and mannitol and did not produce indole. In 1932, Newsom and Cross isolated organisms similar to this group from sheep and cattle and named them *P. haemolytica* (cited by Mutters *et al.*, 1989)

1.1.2 Characteristics of *P. haemolytica*

P. haemolytica is a part of the normal nasal flora of cattle and sheep and is widely distributed in the ruminant population (Gilmour, 1980; Frank, 1989). This species has also been isolated from milk and the female genital tract (Gilmour, 1980; Carter, 1984).

Although bovine respiratory disease is due to a complex interaction of environmental factors or etiological factors that can involve various viral and bacterial pathogens, *P. haemolytica* is one of the most important factors in acute pneumonia in feedlot cattle. It is also the cause of economically important diseases in sheep (Thomson, 1980; Gilmour, 1980). *P. haemolytica* has a cytotoxic effect on leukocytes of ruminants and there are two biotypes and 17 serotypes that are differentiated by their fermentative activity, pathogenicity and serological characteristics.

P. haemolytica is Gram-negative, rod-shaped, non-motile, facultatively-anaerobic, oxidase-positive, indole-negative, urease-negative and shows the property of slight pleomorphism and occasional bipolar staining. A zone of haemolysis is produced around the colonies of fresh isolates. After many subcultures, this zone may be reduced or lost. A double zone of haemolysis on

lamb's blood agar is a characteristic feature. Colonies that grow on blood agar are round and greyish.

P. haemolytica grows on MacConkey's agar and produces small, round, pink to red colonies. Although, the optimum temperature for growth is 37 °C, these bacteria can grow between 25 °C and 40 °C (Carter, 1984). Large numbers of fimbriae and an extensive anionic glycocalyx have been demonstrated at the surface of *P. haemolytica* A1 (Morck *et al.*, 1987).

The first analysis of DNA of *P. haemolytica* revealed that the mol % G+C was 42.3-43.6 (Carter, 1984). However, Lo (1992) showed that the mol % G+C is 38.5%, after analysis of approximately 17 kbp of nucleotide sequence from three regions of the genome of *P. haemolytica* A1. This difference is probably due to certain regions in the DNA of this bacterium containing a much higher mol % G+C.

1.1.3 Biotypes and serotypes

Various techniques have been used to differentiate strains of *P. haemolytica.* For biotyping, the methods have included cultural and biochemical behaviour, antimicrobial resistance (Smith, 1961), gel diffusion (Muraschi *et al.*, 1965), SDS-PAGE patterns (Thompson and Mould, 1975), growth inhibition (Olmos and Biberstein, 1979), agglutination by lectins (Craft *et al.*, 1987) and crossed immunoelectrophoresis (Tsai *et al.*, 1988).

In biotyping based on biochemical and cultural characteristics, *P. haemolytica* has been divided into two biotypes, A and T (these letters stand for arabinose and trehalose fermentation respectively). The A and T types also differ in nucleic acid homology (Biberstein and Francis, 1968) and antibiotic sensivity. Biotype A is more sensitive to penicillin than biotype T. In addition, catalase is produced by biotype A but not biotype T and untypable strains (Mutters *et al.*, 1986).

Lectins are sometimes used for biotype differentiation: wheat germ lectin agglutinates *P. haemolytica* biotype T but not biotype A (Craft *et al.*, 1987).

The principal location of biotype A in the normal host is the nasopharynx whereas biotype T strains tend to be found in the tonsils (Biberstein, 1978). Most septicemic forms of disease in feeder lambs (3-12 months old) are due to biotype T isolates whereas biotype A isolates are associated with pneumonic disease (Biberstein, 1978). Biotype A produces different effects in lambs and sheep. In very young lambs, it produces septicaemia and in adult sheep it causes pneumonia (Gilmour, 1980). These and other differential characteristics of the two biotypes are listed in table 1.

The early serological approaches to the subdivision of biotypes were based on somatic and capsular antigens. The somatic antigens were suggested to be responsible for direct bacterial agglutination by antisera (Biberstein *et al.*, 1960). Using autoclaved whole cells of 98 isolates of *P. haemolytica*, 13 somatic types were recognised. The capsular polysaccharides antigens are presumed to play a role in the indirect haemagglutination (IHA) reaction and these antigens are now commonly used for serotyping (Biberstein, 1978). Strains that cannot be typed by indirect haemagglutination are IHA-negative or untypable, possibly due to a deficiency in capsular polysaccharide. Biberstein *et al.* (1960) reported that approx. 10% of *P. haemolytica* isolates from sheep and cattle were untypable. Seventeen serotypes of *P. haemolytica* have now been distinguished (Biberstein *et al.*, 1960; Pegram *et al.*, 1979; Fraser *et al.*, 1982; Fodor *et al.*, 1988; Younan and Fodor, 1995) and the relationship between biotype and serotype is presented in fig. 1. Thirteen of those serotypes (1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16 and 17) belong to biotype A and four (3, 5, 10 and 15) belong to biotype T.

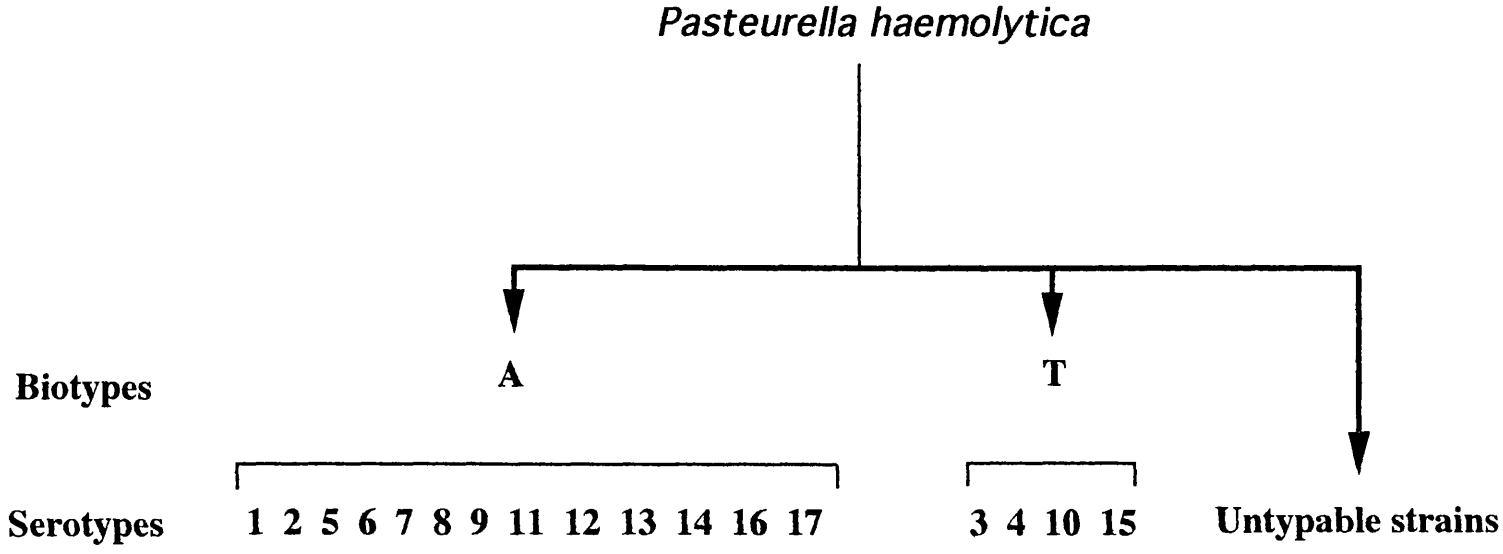
Table. 1

Characteristics of biotypes A and T of *P. haemolytica.*

Characteristics	A	T
Acid production from arabinose and xylose	+	-
Acid production from trehalose and salicin	-	+
Loss of viability of cultures	fast	slow
Penicillin susceptibility	susceptible	relatively resistant
Colonial morphology	small grey	large-yellow-grey
Site of presence in healthy animals	nasopharynx	tonsils
Diseases and hosts	pneumonia in cattle and sheep-septicemia in nursing lambs	septicemia in feeder-lambs
Wheat germ lectin agglutination	-	+

Adapted from Biberstein (1978), Carter (1981; 1984) and Craft *et al.* (1987).

Fig. 1


Pasteurella haemolytica
Biotypes
A
T
Serotypes
1 2 5 6 7 8 9 11 12 13 14 16 17
3 4 10 15
Untypable strains

Although untypable strains can be found in healthy ruminants, they have also been isolated from animals with pneumonia (Ali *et al.*, 1992). However, Gentry *et al.* (1988) showed that representatives of five serotypes 1, 2, 5, 6 and 9, produced lesions when injected into the lungs of healthy cattle but an untypable strain did not cause extensive lesions after injection into the lungs. Untypable strains are found in non-ruminant animals such as rabbits, mice and in other sites of the body such as the genital tract, placenta, udder and intestine in ruminants (Biberstein *et al.*, 1960; Quirie *et al.*, 1986).

Other methods, such as OMP and lipopolysaccharide (LPS) profiles and immunobloting with specific antibody, might help to resolve some of the difficulties in conventional typing methods, especially with untypable isolates. It has been shown that there are clear difference in the OMP and LPS profiles of the different biotypes and serotypes of *P. haemolytica* (Ali *et al.*, 1992; McCluskey *et al.*, 1994). Ten LPS types and 11 OMP types of *P. haemolytica* were distinguished within a collection of serotypes A1, A2 and untypable strains. For example, among the serotype A1 isolates, the majority (19/23) of isolates possessed LPS of type 1, two had type 2 LPS and two had type 3 LPS (Ali, 1993). However, these was no clear correlation of a particular LPS or OMP type in these isolates with the disease status of the host animal.

1.1.4 Plasmids

Plasmids are extrachromosomal DNA structures found in a wide range of bacteria (Novick, 1980) and they are sometimes associated with antibiotic resistance. There are many reports of antimicrobial resistance in *P. haemolytica*. Chang and Carter (1976) showed that about 92% of *P. haemolytica* isolates were resistant to streptomycin, penicillin, tetracycline and chloramphenicol. After a study of 35 isolates *P. haemolytica* from sheep or cattle (most of which are used in this study), Azad *et al.* (1992) reported

that eight isolates (four of serotype A1, three of serotype A2 and one untypable) contained plasmid DNA and isolates of the same serotype had similar plasmid profiles. All plasmid-containing A1 strains exhibited ampicillin resistance which was shown, by transfer studies, to be plasmid-mediated. These strains contained a 4.3 kb plasmid. Strain S/C 84/3 additionally contained two other plasmids of approximately 20 kb and 2.8 kb. The three serotype A2 strains contained two plasmids and an untypable strain contained three plasmids. Neither the T4 nor T10 strains examined contained plasmids.

1.2 *P. HAEMOLYTICA*-THE DISEASES

P. haemolytica is a major cause of pneumonia in cattle and septicaemia in sheep. Other diseases are mastitis in ewes, arthritis and meningitis in sheep and lambs (Gilmour, 1980), and salpingitis in poultry (Carter, 1981). There are a few reports of *P. haemolytica* being isolated from human lesions (Frederiksen, 1989). The bacterium has a low pathogenicity for laboratory animals including rabbits and mice (Carter, 1984) but it can be pathogenic for mice when inoculated intraperitoneally with gastric mucin or alone administered intracerebrally (Smith, 1958).

1.2.1 Disease in cattle

P. haemolytica is found in the upper respiratory tract of healthy cattle (Gilmour, 1978; Wilkie and Shewen, 1988). There are many factors which are thought to result in pneumonic pasteurellosis, including overcrowding, weather, transportation, fasting or other stress and viral disease. These may cause a decrease in lung defences and increase the risk of infection. The most important serotype isolated from cattle with pneumonic pasteurellosis in Britain and North America is A1 (Reggiado, 1979; Gibbs *et al.*, 1983; Quirie

et al., 1986). Other serotypes are reported with less frequency and sporadically (Wray and Thompson, 1971; Frank, 1980; Quirie *et al.*, 1986). Samples collected from the nasopharynx of non-vaccinated cows have shown that serotype A1 was more prevalent than the other serotypes (Frank, 1985).

Fig. 2 shows the probable mechanism of pathogenesis of bovine pneumonic pasteurellosis caused by *P. haemolytica* A1. The organism is frequently found in low numbers in the upper respiratory tract of healthy cattle. Viral infection and stress play important roles in initiating the disease process. The bacteria colonize the mucosal surfaces, survive and multiply. The large number of multiplying bacteria in the nasopharynx can then travel to the lungs. *P. haemolytica* produces both pili and a glycocalyx in the lungs. Pili are surface structures that help the bacterium attach to epithelial cells and thus aid colonization. The glycocalyx is a polysaccharide material that the bacterium produces to protect itself from the host's defence mechanisms (Frank, 1988). The bacteria also produce LPS and leukotoxin (LktA) and these cause lysis of the defence cells which subsequently lead to the production of acute disease with exudative fibrinous pneumonia.

It is well established that macrophages and neutrophils play a predominant role in pulmonary defences against many acute microbial infections and alveolar macrophages produce inflammatory response modifiers. The primary defence of the lung, when the bacteria are present in low numbers, is alveolar macrophages. In addition macrophages secrete interleukin 8 (IL-8), a potent neutrophil chemotactic and activating factor, which causes the migration of neutrophils from the blood vessels to the tissues (Baggiolini *et al.*, 1989). A few hours after the beginning of infection, migration of neutrophils into the lungs has been shown (Walker *et al.*, 1985; Slocombe *et al.*, 1985) and these neutrophils are less resistant to the damaging

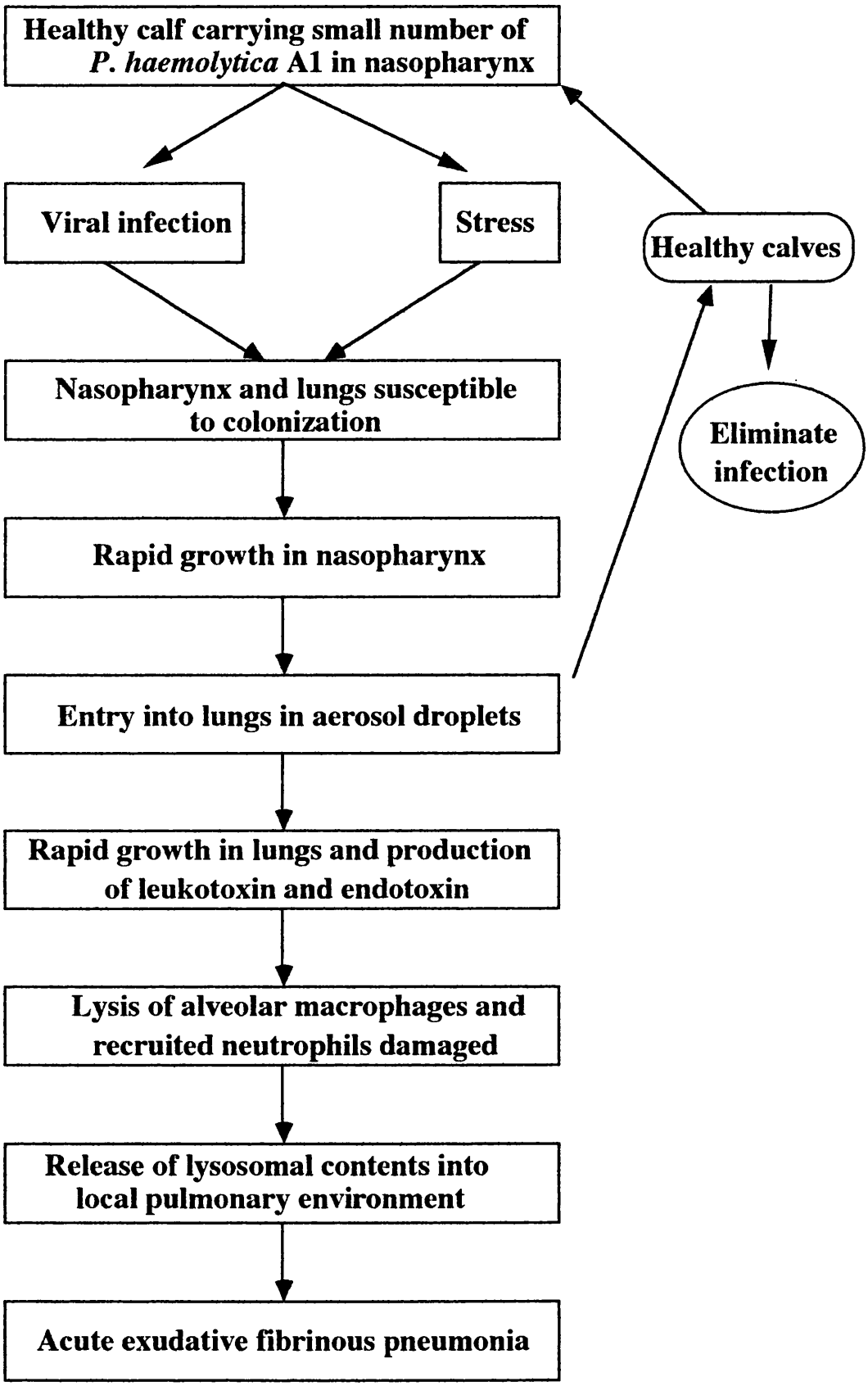
effect of leukotoxin than the resident alveolar macrophages (Czuprynski *et al.*, 1987; O'Brien and Duffus, 1987).

The antimicrobial systems of the neutrophils are classified according to oxygen metabolism. The oxygen-independent agents constitute a group of granule-associated proteins which are released into the phagocytic vacuole and extracellularly (e.g. myeloperoxidase is fungicidal and bactericidal in high concentrations, lysozyme is bactericidal for certain species, lactoferrin is bacteriostatic and proteolytic enzymes are involved in digestion of the foreign particles) (Quie, 1986). The oxygen-dependent systems include several reactive derivatives of oxygen. The initial product of this "respiratory burst" is the superoxide anion. Subsequent reactions lead to the formation of hydrogen peroxide, hypochlorous acid, hydroxyl radicals and singlet oxygen (Rossi, 1986) (see section 1.5.5). It has been shown that the generation of oxygen-derived free radicals and proteolytic enzymes from macrophages and neutrophils in response to infection may cause direct lung injury. Moreover it has been suggested that LktA, in addition to its cytocidal effect on bovine neutrophils, also stimulates a respiratory burst and degranulation of lysosomes (Maheswaran *et al.*, 1992). The release of the lysosomal contents as a result of degranulation can initiate harmful inflammatory reactions and may be responsible for killing of pulmonary endothelial cells and tissue necrosis (Shewen and Wilkie, 1982; Wilkie and Shewen, 1988; Maheswaran *et al.*, 1992; 1993).

P. haemolytica LPS has been shown to induce a variety of clinical and pathological effects when directly instilled into the lungs (Adlam, 1989). There was a decline in the number of circulating leukocytes but lung lavage cell counts were increased with the majority being neutrophils. LPS has

Fig. 2.

Flow diagram showing the mechanism of pathogenesis of bovine pneumonic pasteurellosis caused by *P. haemolytica* serotype A1. Adapted from Frank (1989) and Dalgleish (1990).


Healthy calf carrying small number of *P. haemolytica* A1 in nasopharynx
Viral infection
Stress
Healthy calves
Nasopharynx and lungs susceptible to colonization
Eliminate infection
Rapid growth in nasopharynx
Entry into lungs in aerosol droplets
Rapid growth in lungs and production of leukotoxin and endotoxin
Lysis of alveolar macrophages and recruited neutrophils damaged
Release of lysosomal contents into local pulmonary environment
Acute exudative fibrinous pneumonia

been shown to produce inflammation, oedema, hyperaemia and haemorrhage in the lungs.

Symptoms of pneumonic pasteurellosis include fever (40 °C to 42 °C), dyspnea, fibrinous pneumonia, anorexia, a serous nasal discharge, cough, increased pulse rate, shallow respiration and pleuric sounds in the cranioventral region of the lung (Siegmund *et al.*, 1979). The lesions of pneumonia after natural exposure to *P. haemolytica* are seen in ventral parts of cranial lobes (Jericho, 1989).

1.2.2 Disease in sheep

P. haemolytica produces disease in sheep of all ages and this disease can be acute, hyperacute or chronic depending on predisposing factors. *P. haemolytica* A2 is the most prevalent and pathogenic of all the serotypes causing disease in sheep. This bacterium produces two different disease syndromes: in very young lambs, the septicemic form of pasteurellosis and in slightly older lambs (two to three months), a fibrinous pleurisy with focal lung consolidation has been reported (Gilmour and Gilmour, 1985). However, biotype T produces a septicaemic form in young adult sheep which is associated with a hyper-acute disease (Gilmour 1978) and T strains are cultured from lung lesions in all ages of sheep (Gilmour and Gilmour, 1989).

The clinical signs of pneumonic pasteurellosis in sheep range from sudden death to occasional coughing. Sheep or lambs with pneumonia are dull, anorexic, pyrexic and tachypnoeic or dyspnoeic (Gilmour and Gilmour, 1985).

1.3 VIRULENCE FACTORS

P. haemolytica possesses a variety of putative virulence factors which might be involved at the different stages of the diseases caused by this

organism. These include leukotoxin, haemolysin, capsular polysaccharide, lipopolysaccharide, fimbriae, neuraminidase, various outer-membrane proteins and sialoglycoprotease.

1.3.1 Leukotoxin

Leukotoxin of *P. haemolytica* is said to be target-cell specific and has been reported to act only on ruminant leukocytes. This toxin has been implicated as an important virulence factor which contributes to the pathogenesis of pneumonic pasturellosis by its action on immune effector cells in the lung. More details are provided in section 1.5.

1.3.2 Capsular polysaccharide (CPS)

Polysaccharide capsules are present in most of the species of the family *Pasteurellaceae* and routine methods are used to detect them. The method of Maneval (1941) and the fluorescent antibody technique (FAT) have proved to be better than the other methods for capsular staining of *P. haemolytica.*

The capsule of *P. haemolytica* changes with time, being more abundant in log phase than in stationary phase cultures (Corstvet *et al.,* 1982). Gentry *et al.* (1987), however, showed that in serotype A1, serial passage 128 times did not affect the production of capsular polysaccharide.

For extraction of capsule, various methods have been used, including extraction with water or saline at 56 °C (Carter, 1956; Gentry *et al.*, 1982) and extraction with potassium thiocyanate (Mukkur, 1977) or sodium salicylate (Gilmour *et al.,* 1979). These extracted fractions are contaminated with other substances (Gentry *et al.*, 1982) and for recognising the contaminating substance in the capsule preparation, antibodies are used from sera (Confer *et al.,* 1985) and lavage fluid (Townsend *et al.,* 1987) of vaccinated or experimentally-infected calves.

Adlam (1989) showed that CPS of serotypes A1, A2, A7, T4 and T15 are structurally heterogeneous e.g. A1 CPS is composed of a polymer of N-acetyl-D-mannosaminuronic acid linked 1,4 to N-acetyl-D-glucosamine and is immunogenic in lambs. The CPS of serotype A2 is an α-(2-8)-linked polymer of N-acetylneuraminic acid, but this did not induce an antibody response in lambs (Donachie, 1988).

Injection of purified capsular polysaccharide from serotype A1 has shown that it is immunogenic for sheep but not for rabbits (Adlam *et al.*, 1984). It does not stimulate interleukin-1 by bovine monocytes and is not toxic for neutrophils but purified CPS may affect the phagocytic abilities of alveolar macrophage and neutrophils to engulf the bacteria (Czuprynski *et al.*, 1989; 1991a).

1.3.3 Outer-membrane proteins

In Gram-negative bacteria, the outer membrane is composed of phospholipids, proteins and lipopolysaccharide and plays a crucial role in the survival and multiplication of the organism. Relatively little is known about the structure and function of the outer-membrane protein components of *P. haemolytica*. The major OMPs of serotype A1 (S/C 82/1 isolate) are in the range 30-45 kDa (31, 41 and 43 kDa). Minor proteins of 18, 32 and 98 kDa are also present (Ali, 1993). The profiles obtained by SDS-PAGE are different between the biotypes A and T (Adlam, 1989; Ali, 1993). Variation of OMP profiles within a group of serotype A1 isolates has been reported and these variations could be detected only by western blotting. Variation within serotype A2, however, was detected both by SDS-PAGE and western blotting (McCluskey, 1994).

The OMP profiles of *P. haemolytica* grown under iron-replete and iron-restricted condition have been studied (Deneer and Potter, 1989; Davies *et al.*,

1992). Iron-regulated proteins (IRPs) are located in the outer-membrane of the bacterium and such proteins are often important in bacterial virulence (Weinberg, 1978). The role of IRPs is to help the bacteria in the uptake of iron e.g. bound to host transferrins. Donachie and Gilmour (1988) reported that IRPs of serotype A2 *P. haemolytica* had mol. wts of 70 and 100 kDa. These proteins were found to be present in the outer membrane of *P. haemolytica* A2 cells isolated directly from the pleural fluid of infected sheep. They were expressed only at a low level in A2 cells grown *in vitro* on iron-replete medium. Recently Lainson *et al.* (1991) described the presence of a third IRP of 35 kDa in *P. haemolytica* A2. Similarly, when *P. haemolytica* serotype A1 was grown under iron-limited conditions, additional outer-membrane proteins of 71, 77 and 100 kDa were noted. Immunoblotting of OMPs with convalescent sera from *P. haemolytica* infected calves indicated that antibodies against all three protein were present, suggesting that the proteins were expressed *in vivo* (Deneer and Potter, 1989; Davies *et al.*, 1992).

1.3.4 Lipopolysaccharide (LPS)

Lipopolysaccharide (endotoxin) is located in the outer-membrane of Gram-negative bacteria. It consists of three separate regions; an O-specific polysaccharide chain composed of repeating oligosaccharide units, a low-molecular-weight core oligosaccharide region, composed of approx. 10 monosaccharide units, and a hydrophobic lipid A region (Hitchcock *et al.*, 1986). The core polysaccharide and O-chain polysaccharide are used for serotyping in many organisms and these are antigenic and immunogenic (Rutter, 1988).

Two types of LPS can occur: smooth-type LPS consisting of the lipid A-oligosaccharide core region and O-antigen region, and rough-type LPS

consisting only of lipid A-oligosaccharide core region. Both LPS forms have been shown in *P. haemolytica* isolates.

Until recently, it was assumed that there was a difference in LPS between biotype A and T strains. Biotype A was reported to possess rough-type LPS and biotype T possessed smooth-type LPS (Adlam, 1989). However, the presence of smooth LPS in isolates of the biotype A has been demonstrated by SDS-PAGE and immunobloting (Davies *et al.*, 1991b). Ali *et al.* (1992), after examining 40 isolates of *P. haemolytica* including 23 serotype A1, seven serotype A2, one serotype T4, one serotype T10 and eight untypables from healthy and diseased cattle and sheep, were able to distinguish 10 different LPS profiles by SDS-PAGE and immunoblotting. Davies *et al.* (1992) reported that when two *P. haemolytica* A1 isolates were grown in various conditions *in vitro* such as iron-restriction and different aeration conditions, the LPS profiles did not change markedly.

Endotoxin activity in the culture supernate of *P. haemolytica* serotype A1 grown in non-supplemented medium (RPMI) was initially low, but increased during the growth period. However, in bovine serum albumin-supplemented culture supernates, endotoxin activity decreased with time in culture (Confer and Durham, 1992).

LPS of *P. haemolytica* is known to be toxic for calves and sheep and in pneumonic pasteurellosis is thought to result in increased capillary permeability, thrombosis and coagulation necrosis (Jensen and Mackey, 1979). Different concentrations of LPS have different effects on phagocytosis. At low concentrations, phagocytosis of [^{125}I]-labelled *Staphylococcus aureus* by polymorphonuclear leukocytes was decreased but with higher concentrations, phagocytosis was increased (Confer and Simons, 1986).

1.3.5 Fimbriae

Fimbriae are proteinaceous filamentous appendages of bacteria and are involved in various forms of attachment. In pathogenic organisms, they may have an important role in colonisation of the host. *P. haemolytica* A1 strains produce fimbriae at their surface in the logarithmic phase. Their role in bovine respiratory disease is not clear, but it is has been suggested that they may be important in the colonization of the bovine respiratory tract (Adlam, 1989). There are two types of fimbriae on the surface of *P. haemolytica* A1. The small flexible fimbriae are approximately 5 nm in width and up to 300 nm in length (Morck *et al.*, 1987) and the large rigid fimbriae are approximately 12 nm in width and up to 500 nm in length (Potter *et al.*, 1988). Morck *et al.* (1987) reported that when *P. haemolytica* was grown in shaken culture, they did not find any evidence of fimbriae.

1.3.6 Neuraminidase

Neuraminidase is an enzyme that is reported as a virulence factor of several microorganisms. Scharman *et al.* (1970) first reported that *Pasteurella* species produce this enzyme and found neuraminidase activity in 102 of 104 strains of *P. multocida* and 3 of 5 strains of *P. haemolytica*. However, its role as a virulence determinant in *P. haemolytica* is not clear.

Neuraminidase of *P. haemolytica* serotype A1 has a mol. wt of approx. 150 kDa when analysed by gel filtration and approximately 170 kDa as estimated by SDS-PAGE. The production of neuraminidase increases when the bacteria enter the stationary phase of growth and continues during this phase (Straus *et al.*, 1993). The optimum pH for activity of this enzyme is 6.5. It is stable at 4 °C for 3 h and loses 25% of its activity after 3 h at 37 °C, and 55% of activity after 1 min at 50 °C.

1.3.7 Haemolysin

A complete zone of ß haemolysis is seen around *P. haemolytica* colonies on blood agar plates and this observation is important for differentiating between this species and others. Although all *P. haemolytica* produce haemolysin, the size of zones of haemolysis may differ between different strains. Haemolysin is not dependent on the presence of plasmids (Chang *et al.*, 1987a) and is related to the activity of LktA (see section 1.5.5).

1.3.8 Sialoglycoprotease

Sialoglycoprotease is a proteolytic enzyme and is found in most of the serotypes of *P. haemolytica* (Adlam, 1989). However, the role of this enzyme in the pathogenesis of *P. haemolytica* is not clear. Sialoglycoprotease can release sialoglycopeptides from human RBCs (Otulakowski *et al.*, 1983).

1.4 CONTROL OF *P. HAEMOLYTICA* INFECTIONS

1.4.1 Immunity to *P. haemolytica*

P. haemolytica, like other Gram-negative bacteria, has numerous potential immunogens. Those with the most potential for stimulating immunity include CPS, LPS, OMPs, fimbriae and LktA (Adlam *et al.*, 1984; Shewen and Wilkie, 1985; Morck *et al.*, 1987; Adlam, 1989).

The most studied mechanism of immunity is the humoral immune response which involves a number of complex interactions. Humoral antibody against *P. haemolytica* involves production of antibodies to specific components of the bacterium in addition to LktA-specific antibodies (Confer, 1993). Secretory immunoglobulin A and other factors at mucosal surfaces may inhibit attachment and multiplication of bacteria and thus prevent colonisation of the respiratory tract. These factors may also opsonise the

bacterial cells and enhance phagocytosis and/or neutralize the LktA by binding to leukotoxin.

Information about immune responses to *P. haemolytica* has been obtained from both natural and experimental infections. When cattle are moved to feedyards, the titre of serum antibody to *P. haemolytica* increases both in those that remain healthy and in those that become ill with respiratory disease over a four week period after arrival at the feedyard. However, the antibody titres of the sick cattle increase more than those of the healthy cattle during the first week (Frank, 1989).

Systemic and pulmonary responses of calves to *P. haemolytica* have been evaluated by measuring immunoglobulin production in blood for 9 days and in pulmonary lavage fluid for 7 days after intrapulmonary inoculation. The pulmonary response consisted of production of IgG, IgE, and IgM antibodies to *P. haemolytica*. Antibodies of the IgM isotype to *P. haemolytica* were demonstrated as early as 8 h and for 7 days. Of the anti-*P haemolytica* isotypes, IgM was found in the highest concentration. In all of the inoculated calves, IgE was found 1 to 2 days after inoculation, and IgG was found in 2 of 3 inoculated calves from day 1 through 7 after inoculation. Detection of IgG correlated with smaller pulmonary lesions. However, IgA was not detected in lavage fluid. Specific IgM in serum was detectable 5 days after inoculation, and IgG was detectable 7 days after inoculation. *P. haemolytica* antigens were not detected in serum or plasma. Antigen was detected in lavage fluid by use of monoclonal antibodies raised against *P. haemolytica* CPS, OMP antigens, and LktA in all inoculated calves 8 h after inoculation (McBride *et al.*, 1992).

Tigges and Loan (1993) showed that purified CPS from serotype A1 stimulated production of CPS-specific IgM, IgG1, and IgG2 in calves and predominantly IgM and IgG1 in mature cows. Different adjuvants stimulated different immunoglobulins when mixed with CPS. CPS in oil adjuvant

stimulated the highest mean CPS-specific IgG1 and IgG2 responses, whereas CPS in aluminium hydroxide adjuvant stimulated the highest mean CPS-specific IgM response.

Confer (1993) suggested that because OMPs stimulate homotypic immunity and capsules of various serotypes appear to be serotype specific, LktA may be of major importance for stimulating cross-protection against various serotypes.

In cell-mediated immunity, T-lymphocytes are involved which can act directly on bacterial cells or enhance macrophage killing of intracellular bacteria. However little research has been documented to characterise this particular response to *P. haemolytica.*

1.4.2 Vaccines

Although pneumonic pasteurellosis is thought of as a multifactorial disease, many researchers believe that control of *P. haemolytica* infection would result in a significant decrease in the severity and prevalence of the disease and economic losses caused by it.

Initial attempts to control infections by *Pasteurella* revolved around the use of bacterins (Carter, 1957; Palotay *et al.*, 1963). Results of using such vaccines shown that a formalin-killed *P. haemolytica* bacterin given simultaneously by subcutaneous and aerosol routes resulted in more severe lesions in vaccinated animals than in controls after experimental challenge with *P. haemolytica* (Friend *et al.*, 1977). Combinations of *P. haemolytica* and *P. multocida* have been used with variable success as vaccines. In one field study with such a vaccine, a reduction in respiratory disease was reported (Palotay *et al.*, 1963) but in another study, no significant reduction was found (Martin *et al.*, 1984). Viruses (bovine herpes virus 1 and parainfluenza-3 virus) (Jericho *et al.*, 1976; Mosier *et al.*, 1989) either alone or in combination

with *P. haemolytica* and *P. multocida* bacterin have also been used in vaccines. The results of viral vaccination alone showed little consistent effect in their ability to control shipping fever (Bennett, 1982; Ludwig and Goebel, 1991). In another study, various combinations of viruses and bacterins gave no difference in disease incidence between the vaccinated and controls animals (Mosier *et al.*, 1989).

The use of live vaccines for prevention of shipping fever has shown some promising results. When live *P. haemolytica* was used subcutaneously, intradermally or by aerosol, the reduction of lung lesions and clinical signs of disease shown after experimental challenge compared well with bacterin-vaccinated animal (Confer *et al.*, 1985).

The enhanced protection afforded by most live vaccines compared to bacterins may be due to the stimulation of antibodies to antigens associated with live but not killed organisms (Panciera *et al.*, 1984). Although live vaccines often provide better immunity than non-living vaccines (Confer *et al.*, 1986), vaccines that are produced with live organisms on occasion produce disease symptoms and may only protect against one serotype (Confer *et al.*, 1988). Weekley *et al.* (1993) reported that vaccination with live *P. haemolytica* produced both morphological and functional changes in pulmonary vascular endothelium in sheep. Recently, Petras *et al.* (1995) produced a LktA-deficient mutant of *P. haemolytica* A1 with nitrosoguanidine. When this strain was tested for virulence in goat and cattle challenge experiments, a reduction of mortality and lung lesions was observed in comparison with wild-type strains. Thus, such strains may be suitable live vaccine candidates.

More recent experiments have shown that vaccine efficacy may be dependent on the various adjuvants, dosage and routes of administration used (Mosier *et al.*, 1989). Confer *et al.* (1987) showed that different adjuvants,

including aluminium hydroxide adjuvant (ALH), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA), mixed with *P. haemolytica* bacterins, all stimulated high antibody responses to somatic antigens but low anti-leukotoxin antibody titres. These workers reported that vaccination with killed *P. haemolytica* in Freund's complete or incomplete adjuvant elicited increased resistance to transthoracic challenge, but incorporation of aluminium hydroxide gel as an adjuvant did not. The oil adjuvant bacterin was shown to be reasonably efficacious. The vaccine produced 6-9 months of immunity following initial vaccination in young animals and could protect the animal for 12 months after revaccination (Carter and de Alwis, 1989). This vaccine is viscous and difficult to use, undergoes rapid deterioration at room temperature, has a short storage life and sometimes causes adverse local reactions. Reducing the viscosity of the vaccine usually results in a decrease in immunity compared with the conventional oil adjuvant vaccine.

Intramuscular vaccination of cattle with rLktA or with culture supernate containing LktA has shown that although LktA is an important virulence factor of the organism, an immune response to LktA alone does not protect animals against respiratory disease or against development of post-mortem lung lesions after experimental challenge (Conlon *et al.*, 1991). Confer and Durham (1992) showed that vaccination with the culture supernate of *P. haemolytica* grown in RPMI 1640 medium supplemented with FCS did not effect a change in morbidity of 1140 calves. They suggested that this may have been due to a lack of appropriate surface antigens in the culture supernate.

In an experimental challenge system in SPF lambs, the lambs were challenged with an aerosol of *P. haemolytica* A2 but the challenge was preceded by infection with PI3 virus, a method which consistently induced severe pneumonia in SPF lambs. A crude cytotoxin vaccine of *P. haemolytica* A2 in alhydrogel alone or combined with sodium salicylate extract (SSE)

were tested and the crude cytotoxin vaccine gave 86% protection. A combined SSE with crude cytotoxin gave 98% protection in sheep (Sutherland *et al.*, 1989).

Conlon and Shewen (1991) reported that vaccination of calves with purified CPS was ineffective at protecting calves against *P. haemolytica* challenge. These authors subsequently examined the effects of purified CPS, culture supernate containing LktA, rLktA and various combinations of the above as vaccines. No significant protection was obtained with any factor alone or in combination (Conlon and Shewen, 1993).

Purdy *et al.* (1993) evaluated the effect of various *P. haemolytica* A1 vaccines in goats and reported that only injection of live *P. haemolytica* induced solid protection. However, CPS induced partial protection but LPS and LktA vaccines were ineffective in protecting goats against challenge with *P. haemolytica* A1.

1.5 RTX TOXINS

The RTX (repeats in toxin) cytolytic toxins are a family of toxins produced by different Gram-negative bacteria. They are calcium-dependent and pore-forming and characterised by a series of glycine-rich repeat units containing nine amino acids at the C-terminal end of the protein. The number of the basic repeat units varies between 6 (in LktA of *P. haemolytica*) and 47 (in the adenylate cyclase-haemolysin of *B. pertussis*) (Ludwig and Goebel, 1991). It has been suggested that each toxin is a major virulence factor of its producer organism. The RTX family includes haemolysins produced by members of the *Enterobacteriaceae* (*Escherichia coli* haemolysin [HlyA], *Proteus vulgaris, Proteus mirabilis and Morganella morganii* haemolysins), *P. haemolytica* leukotoxin (LktA), *Actinobacillus actinomycetemcomitans* leukotoxin (AktA), *A. pleuropneumoniae* toxins (ApxIA-III) and *Bordetella*

pertussis adenylate cyclase toxin/haemolysin (cyclolysin) (CyaA) (Coote, 1992). Although there are functional similarities between the different products of the RTX operons, the target cells of the toxins are different. Some of them lyse a wide range of animal and human cells; others are more specific (Shewen and Wilkie, 1982; Cavalieri *et al.*, 1984; Hewlett and Gordon, 1988; Clinkenbeard and Upton, 1991). Target cell specificity is presumably determined by the primary structure and subsequent folding of the toxin which influences its interactions with cell receptors and/or its ability to produce pores in cell membranes of different compositions (Coote, 1992).

E. coli haemolysin (HlyA) is the best characterised member of this family. This bacterium causes urinary tract infections in man and intestinal infections in many species. The toxin has a lytic effect on a wide range of target cells from man and animals. The formation of active extracellular haemolysin requires the products of four *hly* (*hly*CABD) genes that may be located either on the chromosome or on plasmids (Oropeza-Wekerle *et al.*, 1989). Most haemolytic *E. coli* isolates from urinary tract infections or other extra-intestinal infections in humans, and also from the normal human faecal flora, carry the haemolysin genes on the chromosome. However, haemolysin plasmids are present in haemolytic strains from animals (Ludwig and Goebel, 1991).

Lkt is a member of the RTX family (Strathdee and Lo, 1989). The genetic organisation of the *P. haemolytica* leukotoxin operon is similar to that of *E. coli* HlyA (Strathdee and Lo, 1989). It is oxygen-stable, inactivated with trypsin, chymotrypsin and protease (Baluyut *et al.*, 1981; Sutherland and Redmond, 1986; Chang *et al.*, 1987b), pH-stable: range 2-12, heat-labile and inactivated at 100 °C for 30 min or by autoclaving for 15 min but active at 60 °C for 30 min (Sutherland and Redmond, 1986). Lkt has been reported to be unstable at room temperature where 50% of the activity of the toxin is lost in

24 h. However, it appears to be stable at -70 °C for 10 days while storage at 4 °C only maintains toxin activity for 2 days (Sutherland and Redmond, 1986). The maximal production of cytotoxin is reported to be in the logarithmic phase of bacterial growth, at which time the number of viable bacteria is greater than at other phases of growth (Baluyut *et al.*, 1981).

A. actinomycetemcomitans is a gram-negative coccobacillus and is isolated from local juvenile periodontitis and blood samples from patients with endocarditis and abscesses. This bacterium produces a variety of putative virulence factors including a leukotoxin with cytotoxic activity against polymorphonuclear leukocytes and monocytes from man and some monkey species. The toxin is inactive on other cells from man or other species. Although this leukotoxin (AktA) from *A. actinomycetemcomitans* is related to HlyA from *E. coli* (51% amino acid identity), it differs from other members of the RTX family in that it remains cell-associated (membrane bound) and is not secreted into the culture supernate (McArthur *et al.*, 1981; Ludwig and Goebel, 1991; Coote, 1992).

A. pleuropneumoniae is a highly contagious pathogen in pigs, causing pneumonia, and also produces exotoxins that are cytolytic. *A. pleuropneumoniae* is divided into 12 serotypes and produces three different exotoxins in the culture supernate. Two of these toxins possess both haemolytic and cytotoxic activity while the third has cytotoxic but not haemolytic activity. *A. pleuropneumoniae*-RTX-toxin I (ApxAI) is strongly haemolytic and cytotoxic, like *E. coli* haemolysin HlyA, and it has a mol. wt of 105 kDa. It is present in serotypes 1, 5a, 5b, 9, 10 and 11. ApxAII, of 103 kDa, is weakly haemolytic and cytotoxic and resembles *P. haemolytica* leukotoxin LktA. This toxin was found in all serotypes except 10. ApxAIII, of 120 kDa, is cytotoxic but not haemolytic. This toxin is found to be present in serotypes 2, 3, 4, 6 and 8 (Frey *et al.*, 1993; Tu *et al.*, 1994).

Adenylate cyclase toxin (CyaA) is produced by *Bordetella pertussis*, the causative agent of whooping cough. Its enzymic activity is activated by the host factor calmodulin to produce a supra-physiological level of cyclic 3'-5' AMP in target cells. CyaA is a 177-kDa protein and, in addition to adenylate cyclase activity, has weak haemolytic activity. Like active HlyA, the toxin affects a wide variety of target cells (Coote, 1992).

1.5.1 Structure

Four genes are required for synthesis, activation and secretion of the RTX toxins and these are generally arranged and transcribed in the order of *CABD*. The similarity in organisation of the RTX toxin genes in the different species is shown in fig. 3a. Four chromosomal genes *lktCABD* (fig. 3a C) are needed for production of *P. haemolytica* leukotoxin (Strathdee and Lo, 1989). Of these genes, *lktC* and *lktA*, are required to produce an active toxin (Lo *et al.*, 1987) and *lktB* and *lktD* are required for secretion of this protein (Strathdee and Lo, 1989). There are two promoters separated by 258 base pairs and these are located upstream from the *lktC* gene (Strathdee and Lo, 1987; Welch, 1991; Coote, 1992).

The gene encoding the leukotoxin structural protein has been cloned and expressed in *E. coli* (Lo *et al.*, 1985), and its nucleotide sequence has been reported (Highlander *et al.*, 1989). *lktA*, encodes a 102 kDa protein which is inactive without the presence of the 20 kDa *lktC* gene product (Lo *et al.*, 1987).

RTX toxins possess a series of glycine-rich amino acid repeats within the toxin structure and the number of glycine-rich repeat unit varies with different toxins e.g. CyaA has 41 glycine-rich repeats, *P. haemolytica* LktA has 6 repeat units and HlyA has 11 repeat units. Calcium is required for haemolytic activity of active HlyA and interacts with the repeat region. Higher levels of

Fig. 3.

Organisation of RTX toxin genes.

a.

The *A* gene in each case is the toxin structural gene, and this protein is activated by the *C* gene product by an acylation reaction. The activated toxin will be secreted by the *BD* gene products. A. *Bordetella pertussis* adenylate cyclase/haemolysin. The stippled region encodes the adenylate cyclase catalytic region of CyaA. B. *Escherichia coli* haemolysin. The unlinked *tolC* gene is required for secretion. C. *Pasteurella haemolytica* leukotoxin. D. *Actinobacillus actinomycetemcomitans* leukotoxin. E. *Actinobacillus pleuropneumoniae* haemolysin. Arrows indicate the direction of transcription.

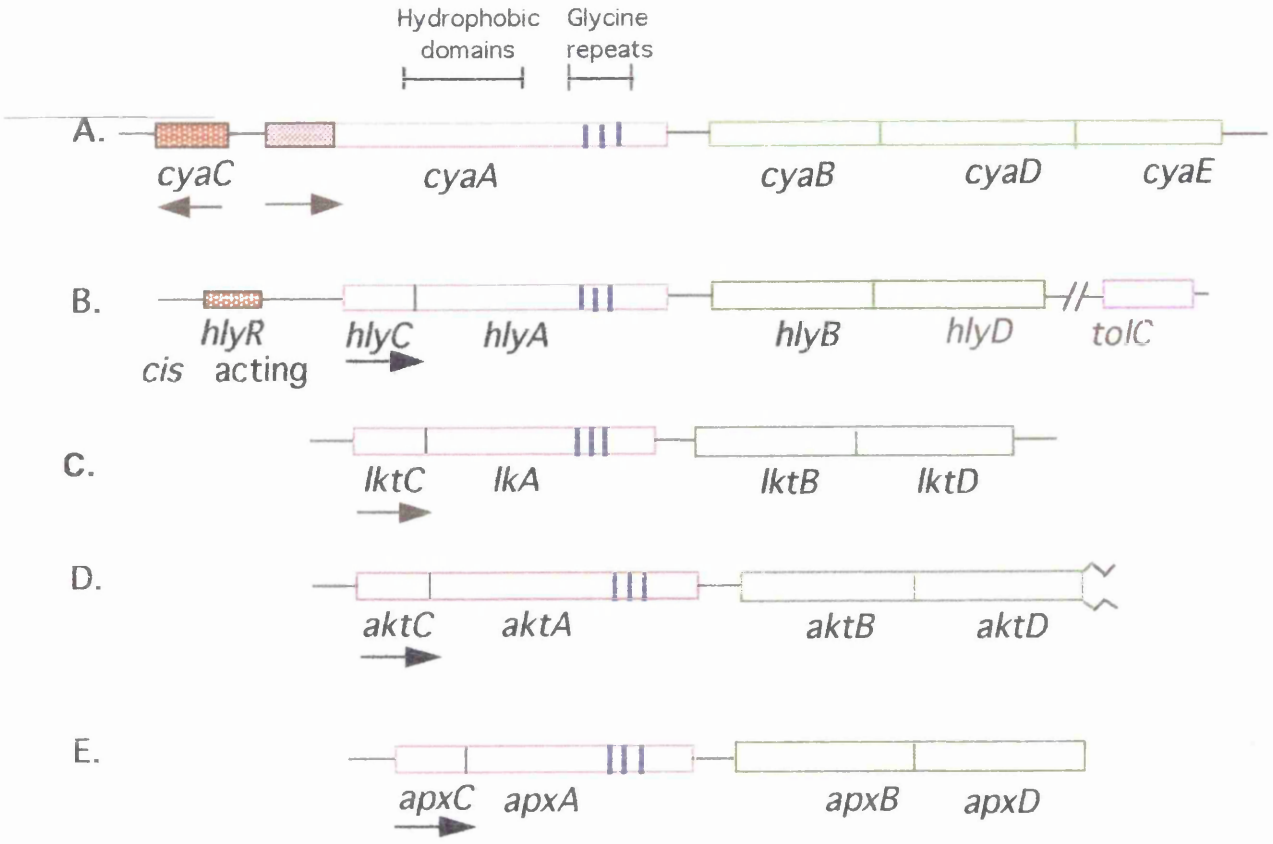
Adapted from Coote (1992); Frey (1995).

b.

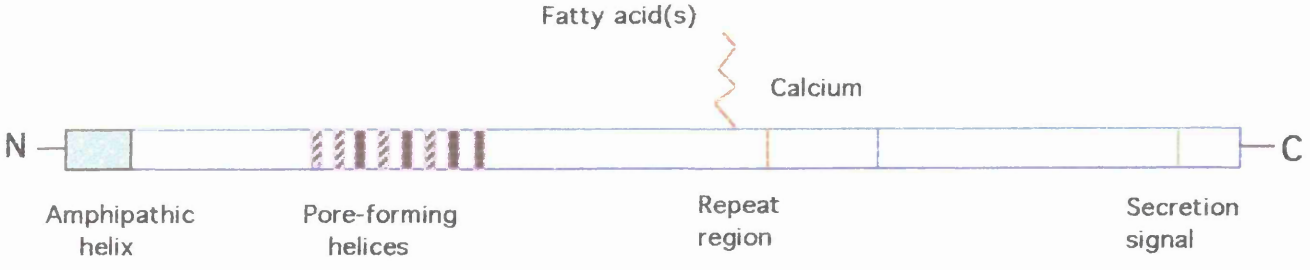
The *E. coli* HlyA haemolysin consists of an amino-terminal amphipathic helix, four amphipathic helices (cross hatched) and four hydrophobic helices (black), which are thought to form the pore in eukaryotic cell membranes, as well as the acylation site(s) (fatty acids), the calcium-binding site and the carboxy-terminal secretion signal.

Adapted from Ludwig and Goebel (1991).

a


Hydrophobic domains
Glycine repeats
A.
cyaC
cyaA
cyaB
cyaD
cyaE
B.
hlyR
cis acting
hlyC
hlyA
hlyB
hlyD
tolC
C.
lktC
lkA
lktB
lktD
D.
aktC
aktA
aktB
aktD
E.
apxC
apxA
apxB
apxD

b


Fatty acid(s)
Calcium
N
C
Amphipathic helix
Pore-forming helices
Repeat region
Secretion signal

calcium were required for haemolytic activity of active HlyA when one repeat unit was removed and the haemolytic activity was abolished by removal of three units (Ludwig *et al.,* 1988; Boehm *et al.,* 1990a). Three regions in active HlyA are involved in pore formation by *E. coli* haemolysin. LktA and LktC share extensive homology (41% and 47% respectively) with HlyA (107 kDa, 1023 amino acids) and HlyC (20 kDa, 170 amino acids) respectively of *E. coli* (Strathdee and Lo, 1987). However, the carboxy terminus of LktA has little homology to that of HlyA (Lo *et al.,* 1987). From the amino acid sequences of LktA and LktC, Lo *et al.* (1987) predicted that LktA would be highly acidic (isoelectric point, 6.27) and LktC would be highly basic (isoelectric point, 9.83). The LktB and HlyB proteins are 80% alike whereas the LktD and HlyD proteins have 60% homology.

Although the mol. wt of LktA from its amino acid sequence is calculated to be 101.9 kDa, the apparent molecular weight was found to be more than 400 kDa by gel exclusion chromatography, 105 kDa as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Chang *et al.,* 1987b) and 104 kDa as judged by size-exclusion high-performance liquid chromatography (Czuprynski *et al.,* 1991b).

1.5.2 Production and activation of RTX toxins

When rLktA was expressed in *E. coli,* both LktA and LktC were detected by immunobloting of urea extracts of the cells with specific antibodies. However, with antisera raised against the soluble antigens in culture supernate of *P. haemolytica* A1, only LktA was detected. So, LktC is an intracellular component and is known to be involved in activation of LktA by chemical modification (Lo *et al.,* 1987). A characteristic of RTX toxins is production of inactive toxin by the *A* gene which then becomes active after modification by the *C* gene product. Post-translational modification and activation of inactive

Hly*A* requires an acyl carrier protein (acyl-ACP) (Issartel *et al.*, 1991) and a number of such cellular proteins contain covalently-bound fatty acids. Myristic and palmitic acid are often covalently linked to proteins, the former by an amide linkage and the latter by ester or by thio-ester linkages. The linkage can be determined on the basis of sensitivity of the lipid-protein linkage to hydroxylamine (Towler and Glaser 1986; Magee, 1990).

The acylation site on HlyA has been located between the pore-forming domain and the calcium-binding RTX motif (fig. 3b). The activity of HlyC is absolutely required for HlyA acylation but its exact function is unknown. It most likely acts as an acyl transferase, but a catalytic activity is difficult to reconcile with the large amount of HlyC required for acylation, which is equivalent to the amounts of HlyA needed. Interestingly, the acylation reaction has been shown not to influence the target cell specificity of the active toxin. For example, *E. coli* with *hlyA, hlyB, hlyD* and *lktC* produces a protein (HlyA) which is neither leukotoxic nor haemolytic. Thus, LktC does not activate HlyA (Forestier and Welch, 1990). However *E. coli* with *lktA* and *hlyCBD* produces a protein (LktA) with weak haemolytic and leukotoxin activity so inactive LktA is activated by HlyC (Forestier and Welch, 1990), but the specificity of the toxin for ruminant cells was unchanged (Highlander *et al.*, 1990). The calcium-binding repeat domain is involved close to the modification site and it is possible that an interaction takes place between these two sites to create the final conformation required for binding and membrane insertion by the toxin (Coote, 1992).

1.5.3 Secretion

After production and activation of the toxin, it must be transported across two membrane barriers (in Gram-negative bacteria) and all RTX toxins, except AktA, are secreted extracellularly. The systems for secretion appear to

be similar. AktA, however, is localized in the periplasm and it can be released into the culture supernate by polymyxin B treatment (Tasi *et al.*, 1984) or by increasing the amounts of $MgCl_2$ (0 to 10 mM) or NaCl (0 to 50 mM) (Ohta *et al.*, 1993). AktA has many basic amino acids (compared to other RTX toxins) which are clustered at the C-terminal end (Kraig *et al.*, 1990) and this may affect its secretion. It has been reported that sequences at the C-terminus of active HlyA are involved in the secretion of protein (Felmlee *et al.*, 1985) (fig. 3b). The secretion signal appears to reside in the C-terminal 38-60 amino acids (Ludwig and Goebel, 1991; Coote, 1992) . Secretion of HlyA does not involve a classical N-terminal secretion signal (Ludwig and Goebel, 1991).

The key elements in HlyA secretion are the translocator protein, HlyB, a member of a superfamily of ATP-dependent membrane-localized transporters, and HlyD, an accessory protein, thought to be required for direct translocation across the cell envelope (Blight and Holland, 1994) (see fig. 4). The carboxy-terminal region of HlyB, which is thought to be located in the cytoplasm, contains two domains that form an ATP-binding site. HlyB most likely transports HlyA through the cytoplasmic membrane at the expense of ATP hydrolysis. In $HlyB^+$ $HlyD^-$ cells, the HlyA protein can be detected by immunogold labelling at the cell surface (Oropeza-Wekerle *et al.*, 1990). HlyD has been proposed to be anchored to the cytoplasmic membrane via its amino-terminal transmembrane moiety while the remainder of the polypeptide is localised to the periplasm (Wang *et al.*, 1991). HlyD could thus form the bridge between the cytoplasmic and outer membranes through which HlyA crosses the periplasmic space. In fact HlyA has never been found in the periplasm, suggesting that it is translocated via fusions of the cytoplasmic membrane and the outer membrane. The HlyA is thus exported directly into the medium without a periplasmic intermediate. When HlyB is absent, HlyA is associated with the cytoplasmic membrane whereas in the presence of HlyB

and N-terminal two-thirds of HlyD, HlyA is associated with both membranes with part of it exposed on the cell surface.

B and *D* gene products are required for export of the LktA and both genes have been expressed in *E. coli* to produce an 80 kDa LktB and 54 kDa LktD protein, respectively (Highlander *et al.*, 1990; Ludwig and Goebel 1991). HlyB and HlyD can be used to secrete the LktA from *E. coli* but secretion of LktA by using LktB and LktD protein in *E. coli* was not successful (Highlander *et al.*, 1989). An additional, outer-membrane protein of *E. coli*, TolC, is required for HlyA secretion from the cell. The *tolC* gene is unlinked to the *hly* operon. Proteins which have homology with TolC have been found in *B. pertussis* (CyaE) (Braun *et al.*, 1993). The specific function of TolC during HlyA translocation is not clear. It might be involved in the formation of fusion sites, or may actually form a pore through which HlyA is translocated through the outer membrane (Braun *et al.*, 1993). In the presence of *hlyA*, *hlyC* and the absence of *hlyB*, *hlyD*, the active toxin accumulates in inclusion bodies and its activity was shown to be the same as that of extracellular active toxin (Oropeza-Wekerle *et al.*, 1989). The accumulation of a relatively large amount of active internal LktA or active HlyA in the logarithmic phase suggests that this cytolytic protein does not damage the membrane of the producing bacterial cell (Oropeza-Wekerle *et al.*, 1989).

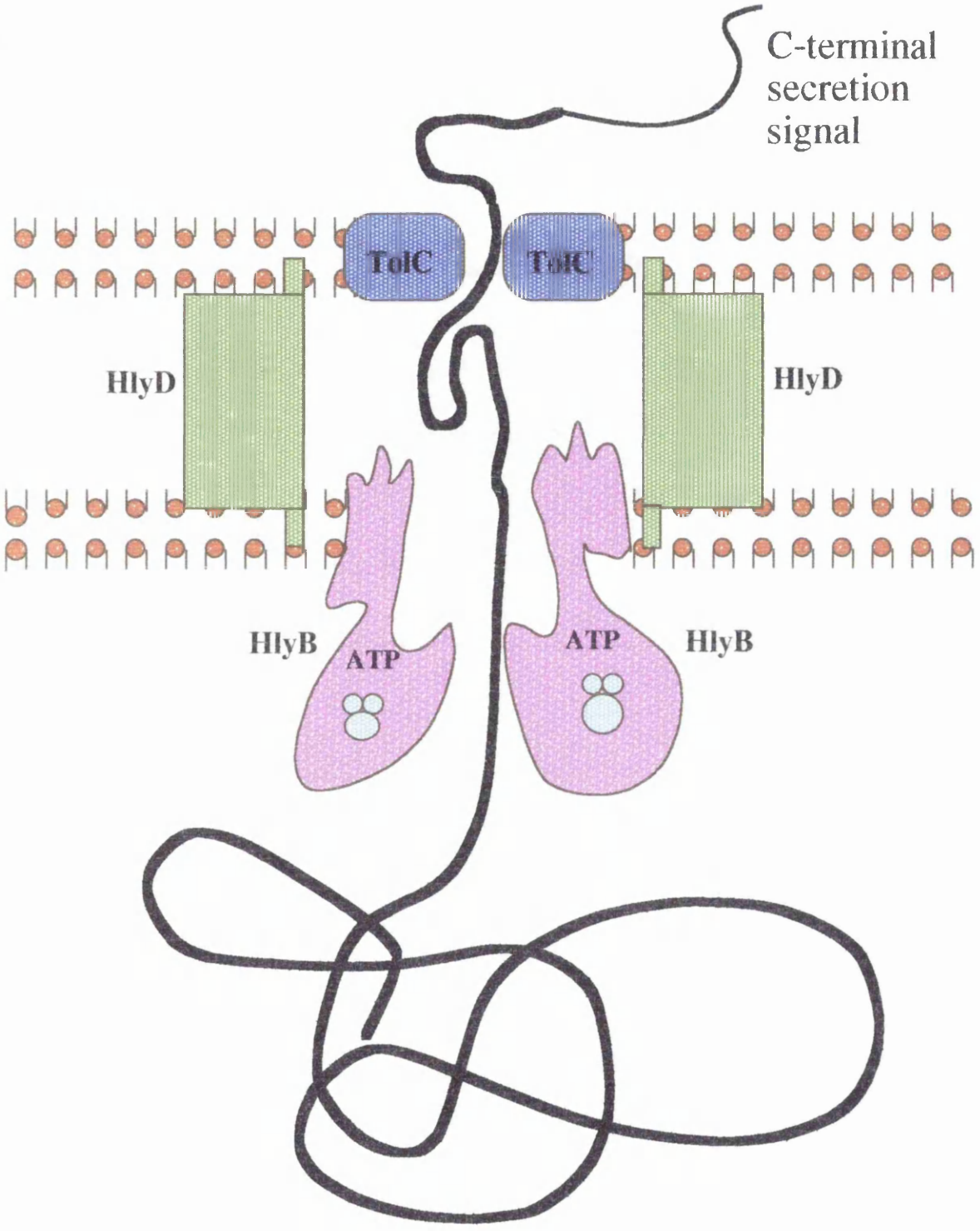
1.5.4 Target cell binding and pore formation

Calcium has been shown to have an important role in the activity of RTX toxins. The calcium can bind to the *hlyA* gene product and the binding site consists of 11 tandemly-repeated glycine-rich units of nine amino acids (residues 739-849) (Braun *et al.*, 1993). Calcium can bind to HlyA in the presence or absence of HlyC (Boehm *et al.*, 1990b). Ludwig *et al.* (1988) suggested that haemolysin-binding to natural membranes is Ca^{2+} dependent.

Fig. 4.

Model of the *E. coli* HlyA translocator complex. HlyB, HlyD and an additional outer membrane protein of *E. coli,* TolC, are required for HlyA secretion into the culture medium.

Adapted from Blight and Holland (1994).


C-terminal
secretion
signal
TolC
TolC
HlyD
HlyD
HlyB
ATP
ATP
HlyB

Rowe *et al.* (1994) reported that lower concentrations of calcium were necessary for lysis of RBC than for BL3 cells. These workers also showed that HlyA could bind to BL3 cells under calcium-deficient conditions that would not allow cytolysis and they suggested that calcium may not be necessary for HlyA to initiate an association with leukocyte membranes (Rowe *et al.*, 1994). However, it was shown that haemolytic activity of active HlyA produced by *E. coli* grown in medium where Ca^{2+} was absent was Ca^{2+}-dependent whereas that produced by *E. coli* grown in the presence of Ca^{2+} was Ca^{2+}-independent. Calcium was required for binding to RBC at both 4 and 37 °C (Boehm *et al.*, 1990a).

It has been suggested that calcium induces a conformational change in CyaA which is necessary for its insertion into the target cell membrane and for maximal intoxication of target cells (Hewlett *et al.*, 1991). Haemolysis of sheep RBC induced by active CyaA occurred in the absence of added Ca^{2+} and in the presence of EGTA (ethyleneglycol-bis(ß-aminoethylether)-*N,N,N',N'*- tetraacetic acid), a calcium chelator, but it is assumed that sufficient Ca^{2+} was sequestered by the toxin during growth of *B. pertussis.*

The influence of calcium on secretion and activity of the cytolysin of *A. pleuropneumoniae* was investigated. *A. pleuropneumoniae* produced toxin in the absence of calcium in the growth medium, but this toxin required calcium both for binding to RBCs and for haemolytic activity (van Leengoed and Dickerson, 1992).

Cruz *et al.* (1990) suggested that both lysis and agglutination of neutrophils was dependent upon the presence of calcium, whereas Clinkenbeard *et al.* (1989b) found that *P. haemolytica* LktA could bind to BL-3 cells in the presence of EGTA. Platelet lysis by *P. haemolytica* A1 culture supernate was found to be Ca^{2+}-dependent and addition of 2 mM calcium to Ca^{2+}-free medium increased the toxin activity whereas addition of 4 mM

EGTA to the buffer containing 2 mM Ca^{2+} decreased toxin activity (Clinkenbeard and Upton 1991). In the presence of EGTA, the release of lactate dehydrogenase by LktA from BL3 cells was blocked but it had no effect on K^+ release or cell swelling (Clinkenbeard *et al.*, 1989b). Active rLktA had lytic activity for BL3 in the presence of 1 mM calcium, and was inhibited by preincubation with 5 mM ethylenediamine tetraacetic acid (EDTA) (Cruz *et al.*, 1990).

It was shown by Oropeza-Wekerle *et al.* (1989) that active extracellular and intracellular HlyA bound to RBCs, whereas the inactive form of HlyA did not bind. Deletion of HlyC or deletion of amino acids 673-726 of HlyA prevented RBC binding, suggesting that in the absence of HlyC modification, HlyA does not bind to RBCs (Rowe *et al.*, 1994). However, it was reported that inactive CyaA can bind to Jurkat cells as effectively as the active form (Hewlett *et al.*, 1993). These differences may reflect differences in methodology used to detect binding

The data from Ludwig *et al.* (1993) indicate that aggregation of two or more HlyA molecules takes place after secretion from the *E. coli* cell and that these aggregates are then able to generate transmembrane pores. The most important part of HlyA for pore formation is the region between amino acids 238-410 of active HlyA, spanning the three hydrophobic subregions (Ludwig *et al.*, 1991) (fig. 3b). Eberspacher *et al.* (1989) showed that binding of *E. coli* haemolysin to erythrocytes occurred both at 0 and 37 °C. However, haemolysis did not occur at the lower temperature. These results indicate that the toxin first absorbs to the cell surface with membrane insertion and pore formation following as a second step. Thus, the process of binding and pore formation by *E. coli* active HlyA are two distinct processes that may be temporally dissociated from each other. It was also suggested that *E. coli*

haemolysin preparations contain a mixture of bindable and nonbindable molecules, the latter being haemolytically-inactive toxin.

Rogel and Hanski (1992) reported that the process of active CyaA penetration into sheep RBCs could be resolved into three consecutive steps: insertion, translocation and intracellular cleavage. Translocation of the NH_2-terminal catalytically-active fragment across the membrane occurred only above 20 °C and was highly temperature-dependent. While a single exposure of the toxin to calcium was sufficient for its insertion into the plasma membrane, toxin translocation required exogenous calcium at mM concentrations. Insertion of the toxin into cell membrane occurred over a wide temperature range (4 °-36 °C). Active CyaA without its C-terminal moiety did not insert into the cells indicating the essential role of this part of the molecule. The N terminus of active CyaA is cleaved inside target cells releasing the active enzyme adenylate cyclase into the cytosol(Rogel and Hanski, 1992).

Rogel *et al.* (1991) showed that intracellular cAMP generation by active CyaA was absolutely dependent on extracellular Ca^{2+} whereas haemolysis occurred in the absence of added Ca^{2+} and the presence of EGTA. These workers suggested that the toxic and haemolytic functions of active CyaA are separable and probably mediated by different domains on the molecule. Similarly, Cruz *et al.* (1990) found that the cell-binding and lytic domains of the leukotoxin of *P. haemolytica* were separable.

Native *A. pleuropneumoniae* ApxIIA toxin is both haemolytic and cytotoxic. When recombinant toxin was expressed in *E. coli,* Tu *et al.* (1994) found two forms of ApxIIA protein. The toxin which remained intracellular had haemolytic and cytotoxic activity, while the secreted toxin was cytotoxic with little or no haemolytic activity. This indicated that cytotoxicity of ApxIIA was independent of its haemolytic activity.

Lysis of cells by RTX toxins presumably occurs by the mechanism described for active HlyA acting on RBCs. It was proposed that lysis of RBCs results from a colloid osmotic shock due to the formation of hydrophilic pores in the plasma membrane which allow movement of small solutes through the pore, whereas large internal molecules cannot pass through (Menestrina *et al.*, 1994).

1.5.5 Production of LktA and effect on target cells

P. haemolytica LktA released from the bacteria may be directly involved in pathogenesis of pneumonic pasteurellosis by impairing pulmonary defences such as alveolar macrophages and neutrophils. All serotypes of A and T biotypes of *P. haemolytica* are reported to produce leukotoxin (Shewen and Wilkie, 1982; Chang *et al.*, 1987a; Burrows *et al.*, 1993). Chang *et al.* (1987a), however, reported that four untypable strains of *P. haemolytica* did not produce leukotoxin. The leukotoxin produced by *P. haemolytica* serotype A1 isolates has been reported to be significantly more active against bovine leukocytes than the leukotoxin produced by *P. haemolytica* biotype T serotype 3 and the latter serotype was shown to produce a toxin which is larger than the A1 toxin, as seen by immunoblotting (Winfield and Lo, 1991). Similar low toxicity has been reported for a serotype A11 strain. The toxic activity of culture supernate of serotype A1 was found to be 50-fold higher than that from A11 (Mdurvwa and Brunner, 1994).

Although LktA is produced by all serotypes (1-16) of *P. haemolytica* and the toxins appeared in similar amounts and to be immunologically related, there are at least seven related *lkt* genes present in these bacteria (Burrows *et al.*, 1993). The seven classes of genes were characterized by their restriction enzyme profiles.

A variety of environmental factors such as temperature, pH, iron and different culture media have been examined for their role in the regulation of *P. haemolytica* LktA (Gentry *et al.*, 1986; Strathdee and Lo, 1989; Confer and Durham., 1992; Gatewood *et al.*, 1994). For example, iron has been shown to be important for the production (Gentry *et al.*, 1986) and secretion of leukotoxin (Czuprynski *et al.*, 1989) and in the absence of free iron in the medium, toxicity is reduced (Strathdee and Lo, 1989).

Baluyut *et al.* (1981) found that the optimum time for maximal toxin production was found to be in the logarithmic phase of bacterial growth. Secretion of leukotoxin started in early log phase and continued until mid-log phase, after which time secretion began to decrease (Strathdee and Lo, 1989). Gatewood *et al.* (1994), however, found that the increasing cytotoxicity in the growing culture could not be explained simply by the increasing number of viable organisms. The production of LktA was more closely associated with growth rate.

Although the secretion of leukotoxin was not affected by growth at 40 °C or 37 °C, it was slightly reduced at 35 °C and was not detectable at 30 °C. Secretion was unaffected at pH 7.3, 7.5, and 8 although at pH 7 and 6.5 the amount of toxin was reduced (Strathdee and Lo, 1989).

P. haemolytica has been grown in different media to study toxin production: brain-heart infusion broth (BHIB) (Strathdee and Lo, 1989); RPMI alone (Kaehler *et al.*, 1980); RPMI containing 0.05, 0.1 and 0.5% bovine serum albumin (Confer and Durham, 1992); RPMI containing 2.5% fetal calf serum (FCS), RPMI containing 700 mg $FeSO_4$, RPMI containing FCS and $FeSO_4$ (Gatewood *et al.*, 1994); or the bacteria have been grown in BHIB for 4.5 h and then in RPMI 1640 containing 7% FCS (Shewen and Wilkie, 1982). The growth curves of bacteria in RPMI alone or with different concentration of BSA were similar. However, the maximum LktA activity

was found when bacteria were grown in RPMI containing 0.5% BSA (Confer and Durham., 1992). Comparing the LktA activity in different media such as BHIB, RPMI, RPMI + FCA and RPMI + FCS + $FeSO_4$, LktA activity was greatest in RPMI containing FCS. However, the growth curves of bacteria in this medium were greater than in the other media (Gatewood *et al.*, 1994).

Gentry *et al.* (1987) found that after 128 passages of *P. haemolytica* serotype A1 in culture, the cytotoxicity and virulence of the bacteria were unaffected.

With regard to target-cell specificity of LktA, it has been reported to affect bovine macrophages (Markham and Wilkie, 1980), bovine leukocytes (Shewen and Wilkie, 1982), bovine neutrophils and bovine lymphoma cells (BL3) (Berggren *et al.*, 1981; Baluyut *et al.*, 1981; Gennaro *et al.*, 1983; Burrows *et al.*, 1993), mononuclear leukocytes (Kaehler *et al.*, 1980), the proliferation of bovine lymphocytes (Majury and Shewen, 1991) and endothelial cells (Maheswaran *et al.*, 1993). Neutrophils are more sensitive than bronchoalveolar or mammary macrophages and peripheral blood mononuclear cells are less sensitive than mammary neutrophils (O'Brien and Duffus, 1987).

There are differences in susceptibility of ruminant neutrophils to active LktA e.g. bighorn sheep neutrophils were four- to eight-fold more susceptible to cytotoxin damage than domestic sheep neutrophils and neutrophils from deer and elk were resistant to killing by active LktA preparations from *P. haemolytica* strains isolated from any species tested (Silflow and Foreyt, 1994). It has been reported that active LktA was not haemolytic for bovine or ovine erythrocytes (Berggren *et al.*, 1981) and that it had no effect on leukocytes from pigs, horses (O'Brien and Duffus, 1987) and man (Kaehler *et al.*, 1980). Other researchers have reported a weak haemolytic activity of the active form of LktA against sheep erythrocytes (Forestier and Welch, 1990;

Burrows *et al.*, 1993). Recently Murphy *et al.* (1995) created a *P. haemolytica* mutant incapable of producing LktA and this strain had neither leukotoxic activity nor haemolytic activity.

The leukotoxin acts on target cells by two mechanisms: firstly causing secretion of K^+ from inside to outside of the cells and secondly by cell swelling. However, leukotoxin does not cause release of K^+, Ca^{2+} uptake or cell swelling in bovine lymphoma 3 (BL3) cells when incubated at 4 °C (Clinkenbeard *et al.*, 1989a; 1989b). After exposure of granulocytes to leukotoxin at 37 °C , cell damage began and, after 5 min, half of the cells, and after 30 min 90% of cells, may be dying. Cooling in ice water was found to decrease or prevent the cytotoxic effect on granulocytes (Styrt *et al.*, 1990). Clinkenbeard *et al.* (1889b) reported that cell swelling was blocked when bovine lymphoma cells or bovine neutrophils (Clinkenbeard *et al.*, 1889a) were incubated in medium with hypertonic sucrose, but Czuprynski and Noel (1990) have shown that various concentrations of sucrose did not affect LktA-induced swelling of bovine neutrophils.

The cytotoxicity of *P. haemolytica* on target cells has been reported using several laboratory methods including electron microscopy, phagocytosis assays, trypan blue dye exclusion, ^{51}Cr-release, colourimetric microtitration assay and luminol-dependent chemiluminescence-inhibition (LDCLI) assays. (Baluyut *et al.*, 1981; Berggren *et al.*, 1981; Shewen and Wilkie, 1982; Chang *et al.*, 1985; Chang *et al.*, 1986a; Vega *et al.*, 1987; Craig *et al.*, 1990). The LDCLI assay was reported to be more sensitive than Cr-release and trypan blue dye exclusion (Chang and Renshaw, 1986).

An interesting effect of active LktA on neutrophils *in vitro* is the activation, at low toxin concentrations, and suppression, at high concentrations, of their respiratory burst. Maheswaran *et al.* (1992) showed that *P. haemolytica* LktA stimulated the neutrophil respiratory burst resulting

in the production of superoxide and H_2O_2. The production of free radicals was seen immediately after incubation with LktA from *P. haemolytica* A1 but, after 5 min, LktA suppressed this respiratory burst and caused cytolysis and degranulation. Czuprynski *et al.* (1991) reported that low concentrations of toxin could stimulate the target cells. No such stimulation, however, was found in a previous study by Czuprynski and Noel (1990), but was in a later study using partially purified leukotoxin. Heat-inactivated (100 °C for 30 min) LktA cannot stimulate the respiratory burst (Maheswaran *et al.*, 1992). High concentrations of active LktA rapidly damage bovine neutrophils (Czuprynski and Noel, 1990).

An effect of LktA on endothelial cells has been reported (Breider *et al.*, 1990). Maheswaran *et al.* (1993) found that crude LktA preparations containing LPS or CPS caused some minimal injury to endothelial cells whereas LPS or CPS alone had no effect. A key factor in endothelial cell killing is involvement of neutrophils. H_2O_2 generated extracellularly from activated polymorphic cells reacts with intracellular iron to form highly reactive hydroxyl radicals (OH) which may cause damage and this may be a key factor in enthothelial cell killing. Comparing culture supernates of *P. haemolytica* A1 and A11, superoxide anion release from bovine neutrophils was greater in response to the culture supernate from an A1 than from an A11 strain (Mdurvwa and Brunner, 1994).

LktA is thought to be an important virulence factor of *P. haemolytica* because of its action on ruminant leukocytes. Target cells are thought to include macrophages and blood leukocytes (granulocytes and agranulocytes). Granulocytes, including neutrophils, eosinophils and basophils, are formed from committed stem cells in the bone marrow and these granulocytes are actively phagocytic and contain myeloperoxidase. Neutrophils are the most common (>60%) of the leukocytes in the blood. It is well estabilished that

neutrophils play an important role in host defence against many acute bacterial and fungal infections. The response of the neutrophils to microbial invasion proceeds sequentially via migration to the infected area in response to chemoattractants derived from both microbial and host sources.

The microbicidal process of phagocytes requires the formation and release of toxic agents to the phagocytic vacuole or extracellularly. The killing mechanism is classified as either oxygen-independent or oxygen-dependent. The oxygen-independent agents constitute a group of granule-associated proteins. The granules contain enzymes which are important for the destruction and digestion of microorganisms and other foreign material. The granules are classified into two types: primary or azurophilic and secondary or specific granules. The azurophilic granules contain peroxidase, myeloperoxidase, acid hydrolases, neutral protease, cationic antimicrobial protease and lysozyme. The oxygen-dependent systems include several reactive derivatives of oxygen. In the neutrophils, when the cell is in the resting phase, the consumption of oxygen is low. However, it is increased when the cells come into contact with activating agents which may be either particulate or soluble. Through the function of the hexose monophosphate shunt, the first carbon of glucose is oxidised to CO_2 via glucose-6-phosphate. Nicotinamide adenine dinucleotide phosphate (NADP) serves as an electron acceptor. By activation of NADPH oxidase by increasing the consumption of oxygen, the electron transfer continues to FAD-flavoprotein which is associated with cytochrome b and from there to oxygen. All of these metabolic events are dependent on activation of NADPH oxidase system which has maximal activity at low pH and catalyses the reduction of O_2 to superoxide (O_2^-) (Badwey and Karnovsky, 1980). Subsequent reactions lead to the formation of hydrogen peroxide, hypochlorous acid, hydroxyl radical and singlet oxygen (fig. 5) (Segal and Abo, 1993). The generation of reactive

compounds of oxygen and proteolytic enzymes from neutrophils in infected lungs may result in direct lung injury and contribute to the pathology associated with pneumonic pasteurellosis (Maheswaran *et al.*, 1992).

1.5.6 Chemiluminescence (CL)

Luminescence is the emission of light and CL is light produced by a chemical reaction. The reactive oxygen products generated by the phagocytes as described above may give rise to natural CL by reaction with cell constituents. This natural CL is only very slight and cannot easily be used in analytical systems but may be potentiated by adding compounds such as luminol, DNDH (7-dimethylamino-naphthalin-1,2-dicarbonic acid) or lucigenin to biological systems. Thus, CL can be used as a measure of phagocytic activity, and interference with phagocytic activity for example by leukotoxicity, and phagocytosis, can be measured as CL-inhibition. Such CL inhibition assays have been used as a sensitive and convenient measure of LktA activity on ruminant neutrophils (Chang and Renshaw, 1986).

The CL response in biological systems may be affected by the presence of contaminating red blood cells and by the composition of the medium such as phenol red and foetal calf serum (Easmon *et al.*, 1980). Several factors may affect the colour and wavelength of the emitted light and these include the type of molecule being excited, the pH of the medium, the presence of metal ions, the type of catalyst and the amount of free energy released by the chemical reaction.

In addition to production of chemiluminescence by phagocytosis of particles such as bacteria or opsonized zymosan, the respiratory burst can also be produced by certain soluble stimuli such as fMetLeuPhe (fMLP)(an example of immature peptide chains from microorganisms) and protein kinase C agonists like phorbol myristate acetate (PMA) (Andrews and Babior, 1984).

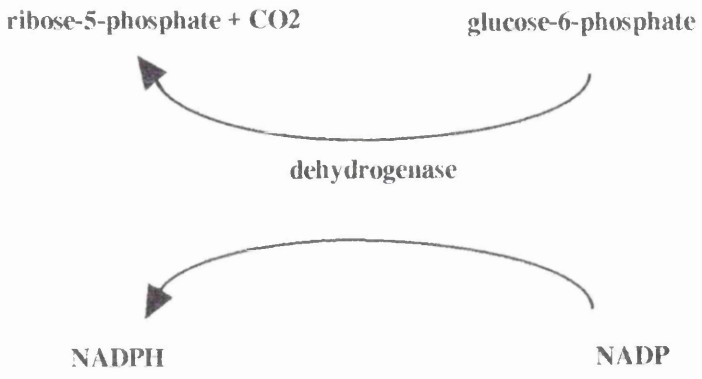
Fig. 5.

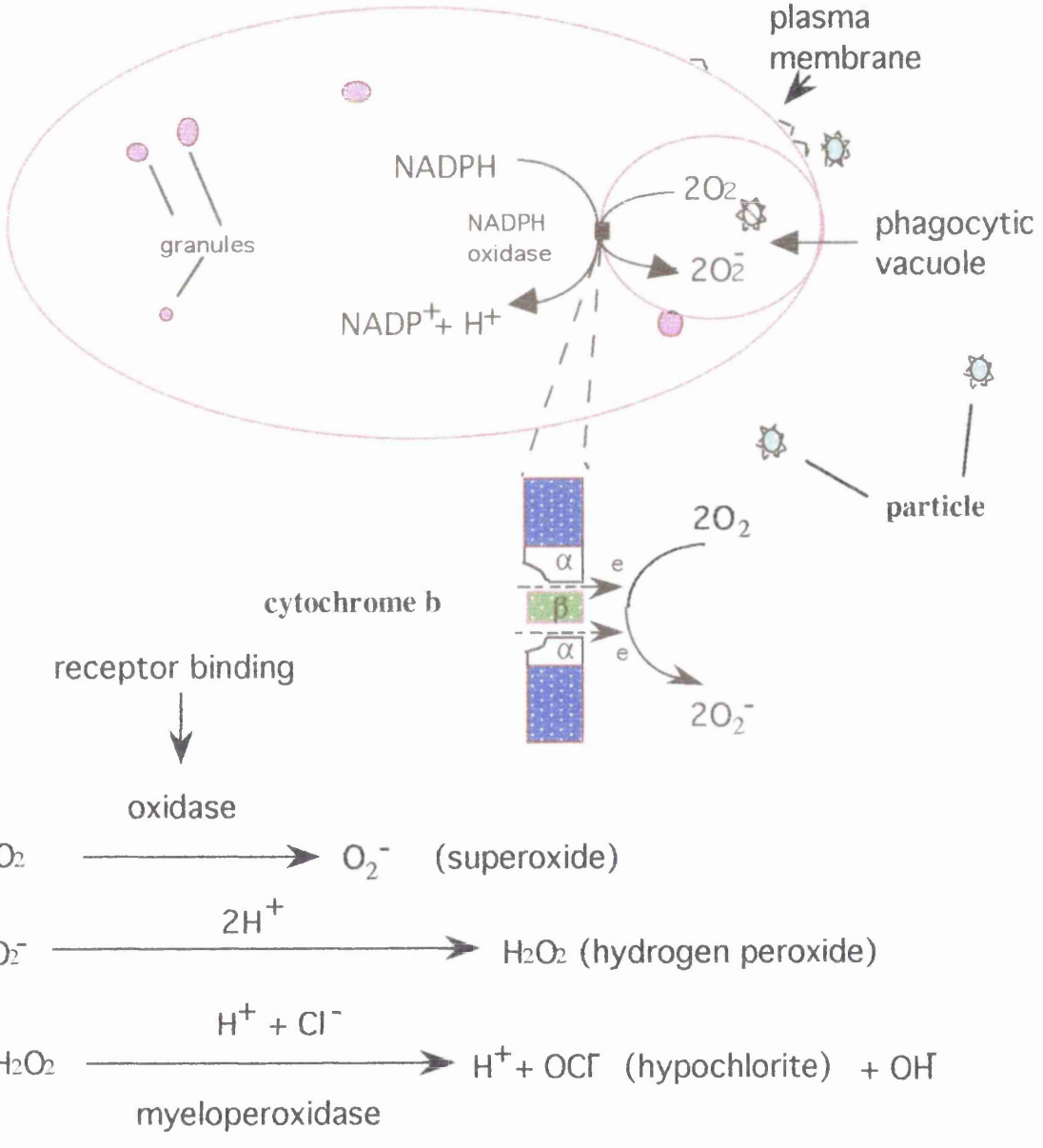
Stimulation of neutrophils and production of free radicals

The respiratory burst is initiated by binding of an opsonized particle to a phagocyte. This binding may initiate ingestion of the particle and activation of an oxidase enzyme. The NADPH oxidase is selectively activated in the wall of the vacuole, resulting in the production of superoxide and hydrogen peroxide within the vacuolar lumen.

Adapted from Rossi (1986) and Segal and Abo (1993).

Hexose monophosphate shunt





fMLP induces a two-peak CL response of human neutrophils, the first peak being due to on extracellular reaction and the second peak due to an intracellular reaction (Briheim *et al.*, 1984). PMA, however, induces a one-peak CL response in normal human neutrophils.

OBJECT OF RESEARCH

Among the many virulence factors produced by *P. haemolytica*, leukotoxin (Lkt) is one which is presumed to have a central role in pneumonic pasteurellosis of cattle and sheep. Lkt has been reported to be specifically lytic for ruminant leukocytes. The main objectives of this study were to compare the production and activity of leukotoxin in culture supernates of different isolates of *P. haemolytica* as judged by SDS-PAGE, immunoblotting and CLI assay and to compare recombinant Lkt produced by *E. coli*, and native Lkt for their effect on different cell types from ruminants and non-ruminants. Another important aspect of Lkt is its homology with other toxins of the RTX family. This study was also aimed at revealing the immunological and biochemical relatedness of Lkt to the other RTX toxins which may help in understanding their structure-function relationships.

2. MATERIALS AND METHODS

2.1 BACTERIA

A description of some of the important characteristics of the *P. haemolytica* isolates examined in this study is given in table 2. All strains prefixed with Ph were collected from various parts of Scotland, from pneumonic and healthy cattle over the period 1982-5. These were kindly provided by Dr. H. A. Gibbs, Veterinary Medicine, Glasgow University, except isolate Ph705, which was provided by Dr. W. Donachie, Moredun Research Institute, Edinburgh. Isolates Ph2, Ph10 and Ph12 were obtained originally from cases of bovine pneumonic pasteurellosis and have been used for experimental infections in cattle to give the typical clinical picture of disease (Gibbs *et al.*, 1984). Isolates Ph26, Ph30 and Ph44 were from the nasopharynx of clinically healthy animals from farms free from disease. Isolate Ph6 was isolated from a healthy animal in-contact with pneumonic cases and later found to be suffering from pneumonia. Isolate Ph14 originated from a confirmed case of bovine pasteurellosis which was slaughtered at Glasgow University Veterinary School. Isolates Ph2, Ph30, Ph42, Ph44 and Ph72 have been used for experimental infection more recently (Davies *et al.*, unpublished). Strains with 5-number designations, i.e. 10632 etc., are from the National Collection of Type Cultures, UK (NCTC strains) and all are ovine isolates. The remainder, a mixture of ovine and bovine isolates, were from the culture collection of Dr. W. Donachie. Many of these strains have been used in previous studies in our Laboratory (Ali *et al.*, 1992; Azad *et al.*, 1992; Davies *et al.*, 1991).

E. coli strains used in this study were obtained from the Laboratory of Microbiology culture collection exept DH5αF'IQ, HMS174 (genotype) and SY327λ pir (genotype) which were obtained from F'*pro*AB+*lac*I^{q}ZΔM15zzf::Tn5[Km^{r}] φ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169

Table. 2.
Characteristics of *P. haemolytica* isolates used in this study.

Laboratory designation	Serotype	Species of origin	Previous* designation	LPS type* PAGE type	LPS type* Chemotype	Isolation	Disease status of host
Ph 2	A1	Bovine	S/C 82/1	1	S	LRT	Pneumonic
Ph6	A1	Bovine	S/C 84/3	-	-	-	-
Ph 8	A1	Ovine	V965B	3	R	Lung	Pneumonic
Ph 10	A1	Bovine	S/B 82/1	1	S	LRT	Pneumonic
Ph 12	A1	Bovine	S/L 82/1	1	S	LRT	Pneumonic
Ph 14	A1	Bovine	H/L 82/1	1	S	LRT	Pneumonic
Ph 26	A1	Bovine	G/A 83/1	2	S	NP	Healthy
Ph 30	A1	Bovine	W/D 83/4	2	S	NP	Healthy
Ph 48	A1	Ovine	FA1	3	R	Lung	Pneumonic
Ph 42	A2	Bovine	D/I 85/1	3	R	Lung	Pneumonic
Ph 44	A2	Bovine	G/T85/15	5	R	NP	Healthy
Ph 72	A2	Ovine	B664	4	R	Lung	Pneumonic
Ph 140	A2	Ovine	T884	3	R	Lung	Pneumonic
Ph 142	A2	Ovine	YS10	3	R	Lung	Pneumonic
Ph 50	A5	Ovine	NCTC 10630	1	-	Lung	Pneumonic
Ph 52	A6	Ovine	NCTC 10632	1	-	Lung	Pneumonic
Ph 54	A7	Ovine	NCTC 10634	2	-	Lung	Peumonic
Ph 56	A8	Ovine	NCTC 10636	3	-	Lung	Peumonic
Ph 58	A9	Ovine	NCTC 10639	(5)	-	Lung	Peumonic
Ph 60	A11	Ovine	NCTC 10642	2	-	NP	-
Ph 62	A12	Ovine	NCTC 10644	3	-	NP	-
Ph 64	A13	Ovine	NCTC 11302	2	-	Lung	Peumonic
Ph 250	A13	Ovine	-	-	S	Lung	Peumonic
Ph 66	A14	Ovine	NCTC 11303	3	-	Lung	Peumonic
Ph 706	A16	Ovine	-	-	-	-	-
Ph 68	T3	Ovine	FT3	-	-	NP	-
FT 4	T4	Ovine	FT 4	-	S	Lung	Peumonic
Ph 252	T10	Ovine	FT10	-	-	-	-
Ph 70	T15	Ovine	NCTC 10641	-	-	-	-
Ph 144	UT	Bovine	UT2	10	R	Lung	Peumonic
Ph 146	UT	Bovine	UT 3	1	S	Lung	Peumonic
Ph 152	UT	Bovine	UT 23	6	S	Lung	Peumonic
Ph 154	UT	Bovine	UT 27	8	R	Lung	Peumonic

*Data from Ali *et al.*, (1992), Ali (1993).
S, smooth LPS; R, rough LPS; -, not known; UT, untypable; LRT lower respiratory tract
NP, nasopharynx

*end*A1 *rec*A1 *hsd*R17(rK$^-$mK$^+$)*deo*R *thi*-1 *sup*E44λ-*gyr*A96 *rel*A1, Gibco BR1, Novagen and Dr, V. Miller, University of California, LosAngeles, USA, respectively. *Pasteurella multocida* NCTC 10322 and *Pseudomonas aeruginosa* NCTC 6749 were from the National Collection of Type Cultures, UK. *Staphylococcus aureus* (delta toxin producer)was kindly provided by Dr. D. Taylor Veterinary Pathology, Glasgow University, and group B streptococcus was kindly provided by Dr. C. G. Gemmell (Department of Bacteriology, Glasgow Royal Infirmary).

Stock culture of the above bacteria were stored frozen at -70 °C in BHIB + 50% glycerol.

2.1.1 Growth of bacteria

The *P. haemolytica* were grown routinely from a glycerol stock on brain heart infusion agar (BHIA) (BHIA, Oxoid, Basingstoke, Hants, UK) containing 5% defibrinated sheep blood (E & O, Limited, Scotland) and incubated overnight at 37 °C. For liquid culture, brain heart infusion broth (BHIB) was used. As part of the characterisation of the *P. haemolytica* isolates, their growth on MacConkey's agar overnight at 37 °C was examined.

2.1.2 Serotyping

To confirm the serotype of the *P. haemolytica* A1 and A2 isolates, the indirect haemagglutination method of Shreeve *et al.* (1972) was used. Briefly, bovine RBCs were obtained from heparinised blood from a healthy animal. Ten ml of blood diluted with phosphate-buffered saline (PBS) was put on the top of 5 ml of Percoll (Sigma) (density 1.092) (table 3) and centrifuged at 1100 x g for 15 min in a bench centrifuge (MSE minor) and the plasma, leukocytes and Percoll were removed. The RBCs were washed three times in PBS pH 7.38 and resuspended in 10 ml of PBS and stored at 4 °C.

Table. 3.

Preparation of different densities of Percoll solution for separation of cells

Stock Percoll:

90 ml Percoll

8.965 ml 10 x HBSS

1 ml 1M HEPES

455 µl 1 N HCl

Density	% Percoll	Volumes added (ml) Stock Percoll	RPMI	*Type of cell separated at given densiy
1.050	38.70	387	613	
1.052	40.39	404	596	
1.054	42.07	421	579	
1.056	43.76	438	562	
1.058	45.45	454	546	Monocyte
1.060	47.13	471	529	
1.062	48.82	488	512	
1.064	50.51	505	495	
1.066	52.19	522	478	Lymphocyte
1.068	53.88	539	461	
1.070	55.56	556	444	
1.072	57.25	572	438	
1.074	58.94	589	411	
1.076	60.62	606	394	
1.078	62.31	623	377	Granulocyte
1.080	64.00	640	360	
1.082	65.68	657	343	
1.084	67.37	674	326	
1.086	69.06	691	309	
1.088	70.74	707	293	
1.090	72.43	724	276	
1.092	74.2	742	258	Erythrocyte

*Percoll solutions of different densities were prepared, bovine blood layered on top and the mixture centrifuged. Seperated cells were examined for the presence of particular cell types.

The antigens for coating the RBCs were prepared from *P. haemolytica* grown overnight with shaking in BHIB at 37 °C. One ml of this culture was transferred to a microcentrifuge tube and heated at 56 °C for 30 min. TheRBCs, 8 x 10^8/ml final concentration, were added to each 1 ml of heated bacterial culture and then incubated at 37 °C in a water bath for 30 min with occasional gentle mixing. The suspension was centrifuged and the pellet washed three times in PBS and resuspended in 1 ml of PBS. Then, 25 µl of sensitised RBCs were mixed in round bottom microtitre plates (Sterilin) with 25 µl of a pre-optimised dilution of type-specific antisera (kindly provided by Dr. W. Donachie), or foetal calf serum or PBS as controls. The plates were incubated for 2 h at room temperature and after this time the haemagglutination reactions were assessed.

2.1.3 API 20 NE assays.

To identify *P. haemolytica* and to distinguish them from other non-enteric Gram-negative rods, the API 20 NE assay (API Montalieu-Vercieu, France) was used according to the manufacturer's instructions. Briefly, the bacteria grown on BHIA overnight at 37 °C were used to prepare an inoculum by suspending 2-6 colonies in 2 ml of 0.85% sterile saline to a turbidity equivalent to 0.5 on the Mcfarland's standard (API#7090) or to an absorbance of 0.125 at 550 nm. The microtubes comprising the conventional tests were carefully filled to avoid the production of air bubbles. The three tests tubes GLU (glucose acidification), ADH (arginine dihydrolase) and URE (urease) were further layered with sterile mineral oil. For the 12 assimilation tests, 200 µl of the bacterial suspension was inoculated into 7 ml of API Auxiliary Medium (which contained (g/l) ammonium sulphate, 2; agar, 1.5; mineral base, 82.8; amino acids, 0.25; vitamins/nutritional substances, 0.0359; in 0.04 M phosphate buffer pH 7.1) homogenised and the microtubes were

filled. Test strips inoculated thus were incubated at 30 °C for 48 h. The assimilation tests for the given substrates were checked for opacity as an indicator of growth and utilisation of that substrate. The results, obtained as scores, were used to generate a profile to match with the Analytical Profile Index (API) designed for non-enterobacteriaceae.

2.2 PREPARATION OF LEUKOTOXIN

2.2.1 Native leukotoxin

All *P. haemolytica* strains were inoculated into 50-ml volumes of BHIB in conical shake flasks and incubated at 37 °C and 120 rpm. After various incubation periods, cultures were centrifuged at 15,000 x g for 15 min at 4 °C. The resulting supernates were passed through 0.45 µm membrane filters (Acrodisc; Gelman Sciences UK), and stored at -70 °C.

Another method used for preparation of leukotoxin was that bacteria were cultured in 50-ml volumes of BHIB for 4 h and then centrifuged at 7,000 x g. The resulting cell pellets were resuspended in 100-ml volumes of RPMI 1640 (Gibco) medium supplemented with 7% v/v foetal calf serum (Gibco) and incubated for 2 h at 37 °C and 120 rpm. Suspensions were then centrifuged at 15,000 x g for 15 min at 4 °C. The supernates were passed through 0.45 µm membrane filters and dialysed against a large volume of distilled water for 24 h at 4 °C. The dialysed supernates were freeze-dried and stored at -20 °C.

P. haemolytica was also grown in Luria-Betani (LB) (see Appendix 4) medium in the presence of 5 µM, 10 µM, 50 µM, 100 µM, 300 µM and 500 µM of EGTA for production of leukotoxin. The EGTA was sterilised by filtration (0.45 µm, Acrodisc) and added aseptically to sterile LB medium.

Growth of bacteria under different conditions of aeration was examined according the method of Davies *et al.* (1992). Briefly, for very high aeration,

50 ml of BHIB was contained in a 250 ml dimpled conical flask; for high aeration, 50 ml of BHIB was contained in a 250 ml smooth conical flask; for moderate aeration, 100 ml of BHIB was contained in a 250 ml smooth conical flask; for low aeration, 200 ml of BHIB was contained in a 250 ml smooth conical flask. All flask were shaken at 120 r.p.m and 37 °C. For no aeration, 200 ml of BHIB was used in a 250 ml smooth conical flask, covered with a layer of liquid paraffin and incubated statically. Bacteria were also grown anaerobically, using an anaerobic jar in conjunction with an anaerobe gas generating kit (Oxoid). Each sachet contains tablets of sodium borohydride, tartaric acid and sodium bicarbonate. When used as directed, they will produce approximately 1800 ml hydrogen and 350 ml carbon dioxide.

2.2.2 Production of recombinant LktA from *E. coli*

Plasmids containing the cloned *lkt* genes (pGW42, pGW64 and pGW78) were constructed and kindly provided by Dr. Gareth Westrop (Laboratory of Microbiology, University of Glasgow). To construct pGW42, a 3.9 kb DNA sequence containing the *lktC* and *lktA* reading frames from *P. haemolytica* A1 was subcloned from recombinant plasmid pLkt5 (Strathdee and Lo, 1989) into vector pIC20H. To construct pGW64 the complete *lktA* gene was subcloned into vector pET11a. Plasmid pGW78 was constructed by first subcloning the *lktC* gene into vector pET11a, to create pGW68. The region of pGW68 containing the T7 *lac* promoter, the translation initiation sequence of T7 gene 10, the *lktC* gene, the T7 transcriptional terminator and the *lac* I gene was then subcloned into pACYC184 to create pGW78.

"Recombinant leukotoxin" is abbreviated "rLeukotoxin" or "rLktA". In *E. coli* strain DH5αF'IQ harbouring plasmid pGW42, transcription of a bi-cistronic mRNA encoding *lktC* and *lktA* is controlled by the *lac* promoter of the vector and is induced by IPTG.

Different strains of *E. coli* (i.e. *E. coli* strains DH5αF'IQ and HMS174 containing plasmid pGW42 (encoding active rLktA) or strain SY327λ pir containing plasmid pGW64 (encoding *lktA*) and/or pGW78 (encoding *lktC*) were used to produce active rLktA.

2.2.2.1 Production of competent cells

E. coli strains (DH5αF'IQ, HMS174 or SY327λ pir) were grown overnight on 2 x YT agar (see appendix 4) plates at 37 °C to give isolated colonies. These were suspended in 50 ml of 2 x YT broth (see appendix 4) to give a starting OD_{650} of 0.02 in a 250 ml conical flask. The cells were grown at 37 °C with shaking (130 rpm) until the OD_{650} of the suspension reached 0.4, when the culture was centrifuged at 2500 x g (Sorval RC5B centrifuge) for 10 min at 4 °C. The cells were then resuspended in 1/10th volume of TSB (see appendix 4) and stored on ice for 10 min. These competent cells were stored in 200 µl aliquots at -20 °C until required.

2.2.2.2 Transformation

For transformation, 2 µl of plasmid DNA (pGW42, pGW64 or pGW78) was added to 200 µl of thawed competent cell suspension and incubated on ice for 20 min and then 800 µl of TSB containing 20 mM glucose was added. The suspension was incubated at 37 °C for 1 h with shaking (130 rpm). Transformed cells (100 µl) were spread on one 2 x YT agar plate containing 100 µg/ml ampicillin (to select bacteria containing pGW42) or 10 µg/ml chloramphenicol (to select bacteria containing pGW64 and/or pGW78) and incubated overnight at 37 °C to give isolated colonies.

2.2.2.3 Growth of recombinant *E. coli* strains

An outline of the procedure for growth of *E. coli* and production of rLktA is shown in fig. 6. The recombinant *E. coli* strains grown overnight on antibiotic plates were inoculated into 250 ml 2 x YT broth containing 100 µg/ml ampicillin or 10 µg/ml chloramphenicol to give starting OD_{650} values of 0.02-0.04. The cells were grown at 37 °C with shaking. At OD_{650} of 0.5, IPTG (Sigma) was added to the medium at a final concentration of 1 mM to induce expression of the recombinant proteins. The culture was incubated for a further 4 h at 37 °C. The cells were harvested by centrifugation at 9,000 x g for 10 min at 4 °C and the pellets were resuspended in 1/10 volume of ice-cold TEN buffer (see apendix 4), and stored at -20 °C in aliquots.

2.2.2.4 Preparation of urea extract.

Various methods were used for lysing the cells:

1. A 10 ml volume of cell pellet was thawed at 37 °C, 1 ml of lysozyme (10 mg/ml) was added and the mixture incubated at 4 °C for 1 h. After this, 20 ml of TEN buffer containing 1.5% sodium deoxycholate was added and the cells were then centrifuged at 17,000 x g for 10 min at 4 °C. The cells were resuspended in 30 ml TEN buffer containing 0.5 % sodium deoxycholate to lyse the cells and disrupt the DNA and centrifuged as above.

2. The cell pellet was suspended in TEN buffer (see appendix 4) containing lysozyme (10 mg/ml) and glycerol, and kept for 1 h at 4 °C and then centrifuged at 17,000 x g for 10 min at 4 °C.

3. The cell pellet was suspended in Hanks Hepes (HH buffer) (see appendix 4) and sonicated at 4 °C and then centrifuged at 17,000 x g for 10 min at 4 °C.

4. It was found that a combination of methods 2 and 3 gave better lysis of cells.

The resulting pellet of broken cells and inclusion bodies was suspended in 30 ml of distilled water and washed three times. The protein was extracted by suspending the pellet in 1 ml of TC buffer (see appendix 4) containing 8 M urea prior to rotation at 4 °C for 1 h. The pellet and insoluble material were separated by centrifugation at 15,000 x g for 20 min at 4 °C. The urea extract (supernate), containing the recombinant leukotoxin, and urea insoluble fraction were stored at -20 °C.

2.2.2.5 Dialysis

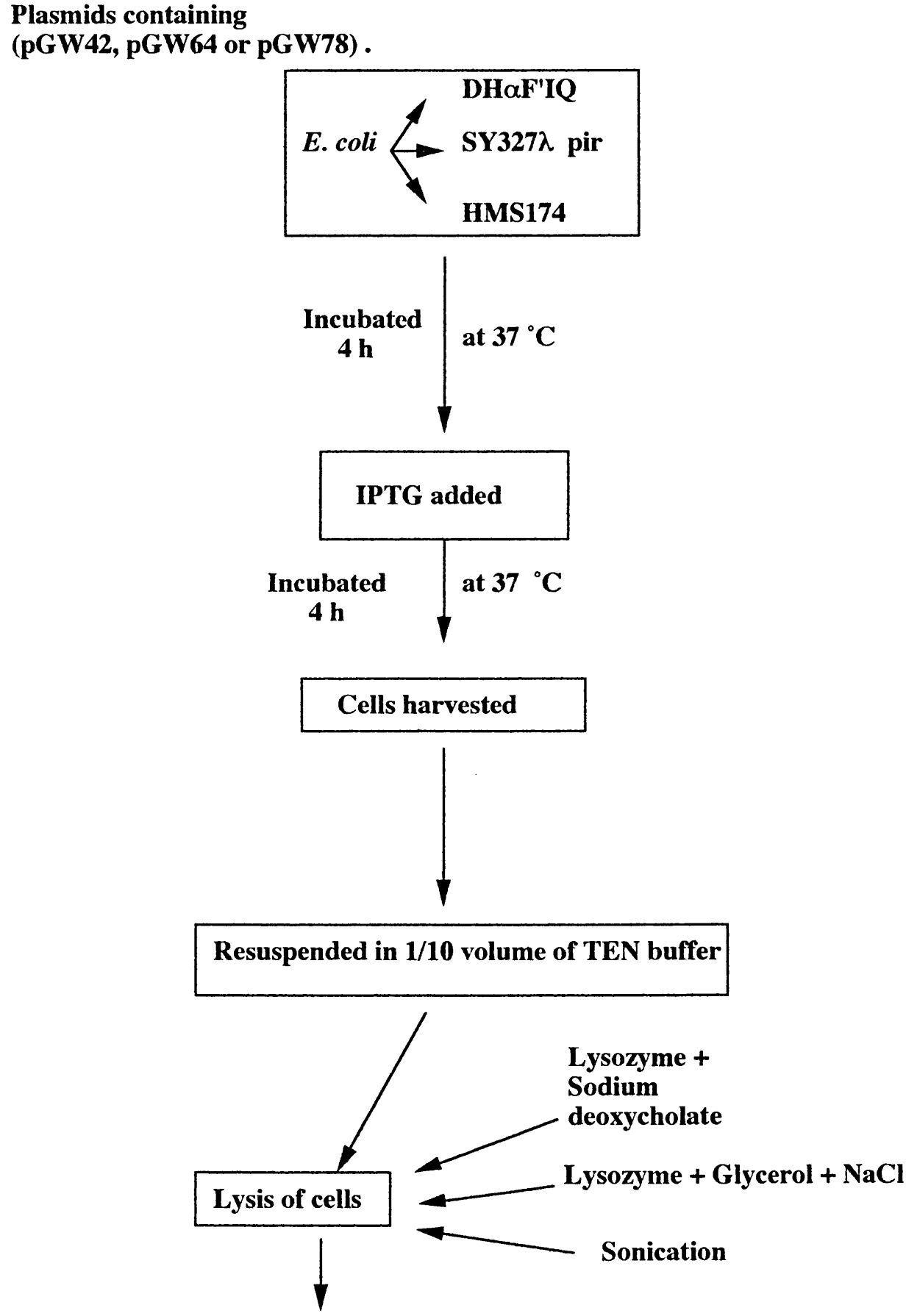
Urea was removed by dialysis of the urea extract against a large volume of buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$) or HEPES buffer (25 mM) for production of toxin in absence of calcium. Dialysis was done immediately before any toxicity assay, according to the method of Brownlie *et al.* (1988). Urea extract was placed in a dialysis membrane (size 1-8/32") and dialysed at 4 °C for 2 h with continuous mixing. The dialysis buffer was changed twice during this time. After dialysis, the samples were transferred to microcentrifuge tubes, and stored on ice until used.

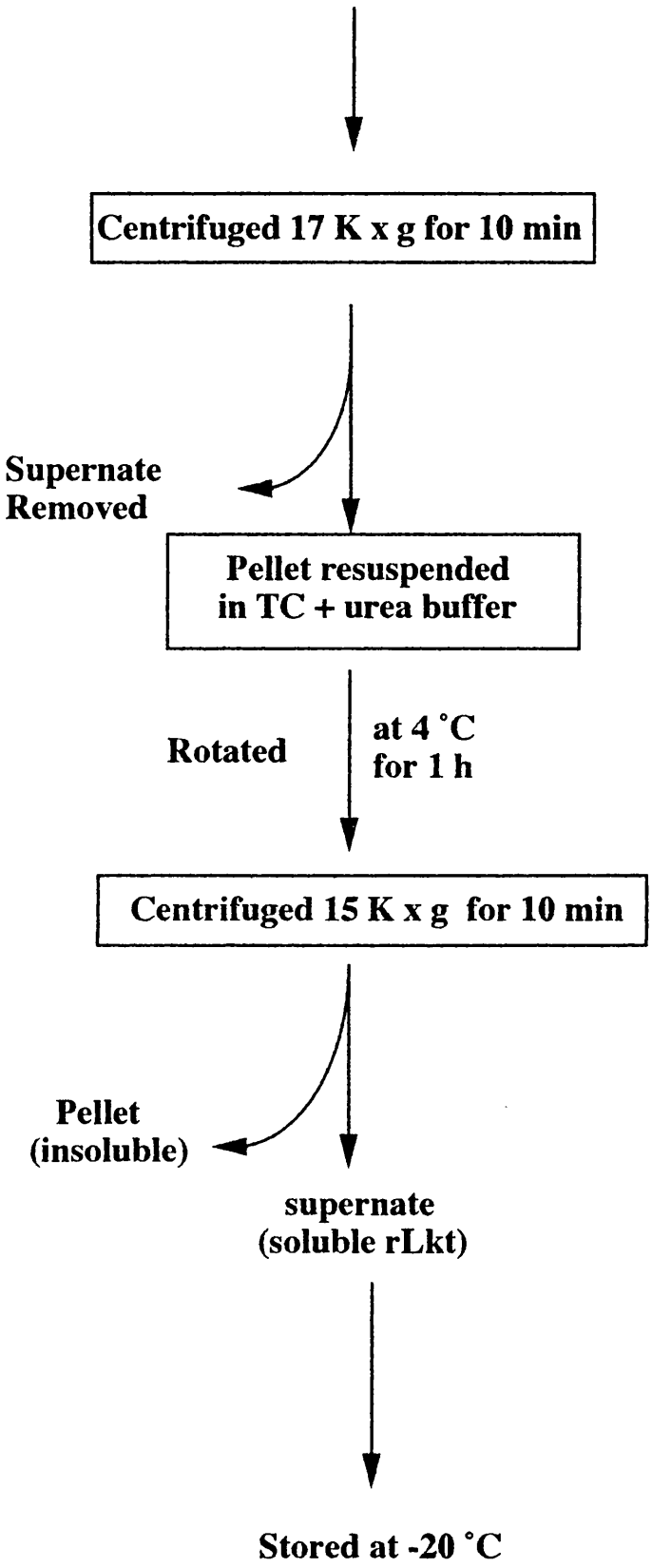
For production of rLktA in the absence of calcium, *E. coli* SY327λ pir containing pGW42 was grown in LB medium in the presence of 100 μM EGTA. No calcium was added at any step in the extraction procedure.

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

SDS-PAGE was according to the method of Laemmli (1970) and the reagents used are described in Appendix 3. The electrophoresis was performed in a vertical slab gel apparatus (Protean II; BioRad). Stacking gels contained 4% w/v acrylamide and resolving gels contained 10% w/v acrylamide, respectively. Samples were solubilised in equal volumes of sample buffer and heated in a water bath at 100 °C for 5 min. 100 μl volumes of

Fig. 6. Production of rLkt from *E. coli*





Urea was removed by dialysis or dilution before use

solubilised culture supernate samples were loaded per well. Molecular weight standards (SDS-6H, Sigma) were treated in the same way. Samples were electrophoresed into the stacking gel at 20 mA and then at 25 mA in the separating gel for 4-5 h or until the tracking dye reached the bottom of the gel. The gel was then stained with coomassie blue R-250 (Sigma) staining solution for 1 h and destained by several changes of destaining solution.

2.4 WESTERN BLOTTING (IMMUNOBLOTTING)

After SDS-PAGE, the resolved protein bands were transferred to nitrocellulose membrane (Hybond-C super, Amersham) according to the method of Towbin *et al.* (1979) but with slight modification, in a Bio-Rad transblot cell. The transfer buffer consisted of 192 mM glycine, 25 mM Tris (pH 7.3) and 20% v/v methanol. Proteins were transferred at 15 volts, with cooling, for about 15 h, followed by 30 volts for 2 h in order to transfer the high molecular weight proteins. The nitrocellulose membrane was stained briefly (3 min) with 0.5% Ponceau S in 0.1% v/v acetic acid in distilled water, then destained with three changes of distilled water. The positions of transferred protein bands were visualised and those of the mol. wt standards were marked. PBS was then used for complete destaining. Non-specific binding sites were blocked for 1 h at room temperature with gentle shaking in PBS containing 0.1% Tween 20 and 5% w/v dried skimmed milk. The membrane was then washed twice for 5 min with fresh changes of PBS containing 0.1% Tween 20 at room temperature. Blots were probed with primary antibody diluted in PBS and incubated for 1 h at room temperature. After further washing as above, an appropriate HRP-conjugated secondary antibody diluted in PBS was added and incubation was for 1 h at room temperature. The nitrocellulose membrane was washed and then developed with a mixture of 0.03% w/v DAB (3-3 diaminobenzidine tetrahydrochloride

dihydrate), 98 ml PBS, 2 ml of aqueous 1% w/v $CoCl_2$ and 100 µl of hydrogen peroxide. The reaction was stopped with distilled water and the membranes were stored in the dark.

2.5 CHEMILUMINESCENCE (CL) ASSAY METHODS

2.5.1 Isolation of leukocytes

Heparinised blood was obtained from healthy humans, rabbits, guinea pigs, calves and sheep. For preparation of neutrophils, 10-ml volumes of blood were diluted with equal volumes of PBS and carefully layered on top of 7 ml of Histopaque (Sigma) 1.077 g/ml and centrifuged in polystyrene plastic universals at 830 g for 30 min at room temperature. The supernatant layers containing plasma, mononuclear cells and Histopaque were aspirated. For lysis of the red blood cells, the pellet was suspended in 3 volumes of ice-cold NH_4Cl medium (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). After 5-7 min, the colour of the cell suspension changed from red to black indicating lysis of the erythrocytes. The suspension was then centrifuged at 400 g for 5 min at 4 °C. The lysis procedure was repeated and the remaining cells centrifuged twice with PBS and finally suspended in PBS or HH (pH 7.38). The resulting cell suspension was >95% neutrophils. Neutrophil viability, determined by 0.1% trypan blue dye exclusion was >98% in all preparations examined.

Another method used for preparation of neutrophils, lymphocytes and monocytes was to use Percoll at different densities shown in table 3. The stock Percoll was diluted with RPMI and the density giving optimal separation of each cell type was determined. For example, for preparation of neutrophils, the diluted blood was carefully layered on the top of 7 ml of Percoll (Sigma) 1.092 g/ml (table 3) and centrifuged in polystyrene plastic

universals at 830 g for 30 min at room temperature. The supernatant layers containing leukocytes and plasma were aspirated and carefully layered on top of 7 ml of Histopaque (Sigma) 1.077 g/ml and centrifuged in polystyrene plastic universals at 830 x g for 30 min at room temperature. The neutrophils were separated from mononuclear cells and washed two times with PBS and finally suspended in PBS or HH. The resulting cell suspension was >85% neutrophils. Neutrophil viability, determined by 0.1% trypan blue dye exclusion was >98% in all preparations examined.

A bovine leukaemia (BL3) cell line and a mouse macrophage cell line (J774.2) were kindly provided by Dr. G. Westrop.

2.5.2 Opsonization of zymosan

For preparation of opsonised zymosan, 100 mg of zymosan A (Sigma z-4250, from *Saccharomyces cerevisiae*) was added to 5 ml of normal bovine serum, 10% v/v in PBS, and this mixture was incubated at 37 °C for 30 min with shaking at 30 rpm. The opsonised zymosan was washed twice with ice-cold PBS and finally suspended in 10 ml in PBS, and stored at -20 °C in aliquots until used.

2.5.3 CL assay reagents and buffers

DNDH (7-dimethylamino-naphthalene-1, 2 dicarbonic acid hydrazide), luminol (3-aminophthal hydrazide) (Sigma) and lucigenin (bis-n-methyacridinium nitrate) (Sigma) were prepared as 10^{-2} M stock solutions in DMSO (dimethyl sulphoxide). The working dilutions (10^{-4} M) were prepared in PBS or HH. Phorbol myristate acetate (PMA) (Sigma) stock was prepared at 2 mg/ml in DMSO and used to prepare the working solution at 10 µg/ml in PBS. fMLP [formyl methionine-leucine-phenyl-alanine (Sigma)] was prepared as 10^{-5} M stock in PBS and was used as 10^{-7} M final concentration in the

assay.

The usual CL assay procedure was to mix 15 µl of culture supernate containing leukotoxin with 785 µl of HH containing 5 x 10^5 neutrophils and 100 µl of a working dilution of chemiluminogenic probe and incubated for 20 min at 38 °C. Neutrophils were then stimulated with 100 µl of OZ. CL was measured with a Wallac luminometer connected to a IBM-PC computer with Multi-use software (Bio-Orbit). The chemiluminescence emission was measured in millivolts (m. v) at 38 °C. Each sample was tested in duplicate and contained neutrophils (5 x 10^5), DNDH (10^{-5} M), OZ (0.5 mg) or PMA (1µg), PBS or HH(pH 7.38) and the final volume was 1 ml. The assay was performed in duplicate and the mean value calculated.

2.6 ANTIBODIES

2.6.1 Preparation of rabbit polyclonal antibody to rLktA

Recombinant LktA obtained from urea extracts of *E. coli* was resolved by SDS-PAGE using a 7.5% acrylamide separating gel. The gel was stained with coomassie blue R 250 for 5 min and then destained for 20 min. The bands at 100-110 kDa were carefully cut out. Precautions were taken to handle the material carefully with gloved hands and alcohol-sterilised scalpel and forceps to avoid contamination. Gel bands were washed in sterile PBS, crushed, suspended in 2 ml sterile PBS and stored at -20 °C. One day before injection of protein, the sample was placed in narrow dialysis tubing and dialysed against large volumes of saline (mixed occasionally) for 24 h. The gel-free supernate was emulsified in Freund's incomplete adjuvant (Sigma) in 1:1 ratio. Two female New Zealand Albino rabbits were injected with 2 ml at two sites either subcutaneously or intramuscularly. Before injection of sample, the rabbits were bled for pre-immune sera. The immune sera were collected after 4

booster injections at monthly intervals. The procedure is outlined in fig. 7.

2.6.2 Monoclonal antibodies

Mouse ascitic fluid and hybridoma culture supernate containing monoclonal antibody (MAb) raised against rLktA was kindly provided by Dr. W. Donachie, Moredun Research Institute, Edinburgh, UK.

Ascites fluid containing monoclonal antibody (9D4), (IgG2a), raised against *Bordetella pertussis* adenylate cyclase toxin (CyaA) was kindly provided by Dr. E. Hewlett, University of Virginia, USA. This MAb reacts in immunoblots with the 210 kDa band of CyaA as well as with the 105 kDa band of LktA.

Monoclonal antibodies GJMC-1 and GJMC-3 raised against the ApxAIII toxin of *Actinobacillus pleuropneumoniae* were kindly provided by Dr. J. Cullen, Department of Veterinary Pathology, Glasgow University. These monoclonal antibodies react strongly with the 120 kDa protein and also with the other 2 Apx toxins (ApxAI & ApxAII) to a lesser extent. They also cross-react with products from *E. coli, A. suis, P. haemolytica,* and *A. equuli* (J. Cullen, Ph. D. Thesis, University of Glasgow.

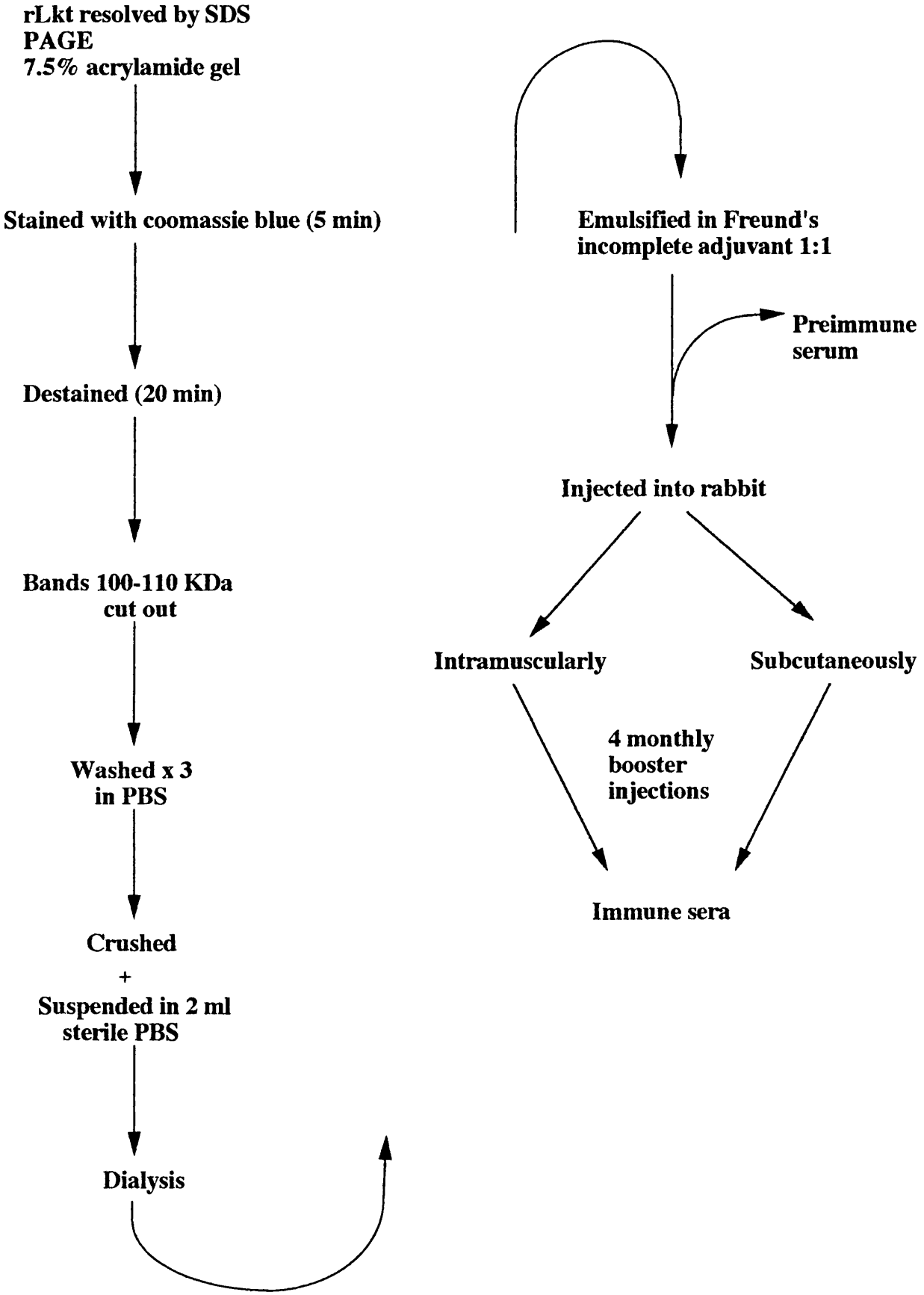
Monoclonal antibody 2-5 (IgG1) raised against AC toxin was kindly provided by Dr. N. Heveker, Institute of Pasteur, Paris. This MAb reacted with the repeat region of the AC toxin.

2.6.3 Other antibodies

Rabbit polyclonal antibody raised against purified native AC toxin was kindly provided by C. Brotherston, Laboratory of Microbiology, Glasgow University.

Rabbit polyclonal antibodies raised against the 200 kDa band of CyaA, and against CyaC (21 kDa) cut out from SDS-PAGE, were kindly provided by

Fig. 7. Preparation of polyclonal antibody to rLkt



E. K. Hormozi, Laboratory of Microbiology, Glasgow University.

Rabbit polyclonal antibodies raised against phenol-purified LPS from *P. haemolytica* strains Ph2 and Ph30 were kindly provided by Dr. R. L. Davies, Laboratory of Microbiology, Glasgow University.

Convalescent serum from a calf experimentally infected with *P. haemolytica* strain Ph2 and used by Dr. Q. Ali (Ph. D. Thesis, Glasgow University) was also available.

2.7 HAEMOLYSIN ASSAY

Haemolytic activity of *P. haemolytica* leukotoxin and urea extracts of rLktA from *E. coli* was determined as follows. For preparation of RBCs, 10 ml of sheep blood diluted 1:1 in PBS or HH was layered on top of 5 ml Percoll with a density of 1.092 in a 15 ml centrifuge tube and centrifuged at 1200 x g for 30 min. Plasma, leukocytes and Percoll were removed and the pelleted RBCs were washed in PBS or HH until the supernate was clear. The RBCs were resuspended in HH buffer containing 1 mM $CaCl_2$. Serial two-fold dilutions of culture supernate from *P. haemolytica* or dialysed urea extracts containing rLktA of *E. coli* (active and inactive form of toxin) were mixed with equal volumes of 4 x 10^9 RBCs in a 96-well flat bottom microtitre plate and incubated overnight at 37 °C. The RBCs were mixed with BHIB or urea solution diluted in HH respectively as controls. After this incubation, the RBCs were pelleted by centrifugation at 1500 x g and the supernates transferred into a clean plate and the amount of released haemoglobin in the supernate was determined spectrophotometrically at 540 nm in an Anthos 2001 ELISA reader. The assays were performed in duplicate and the mean values calculated.

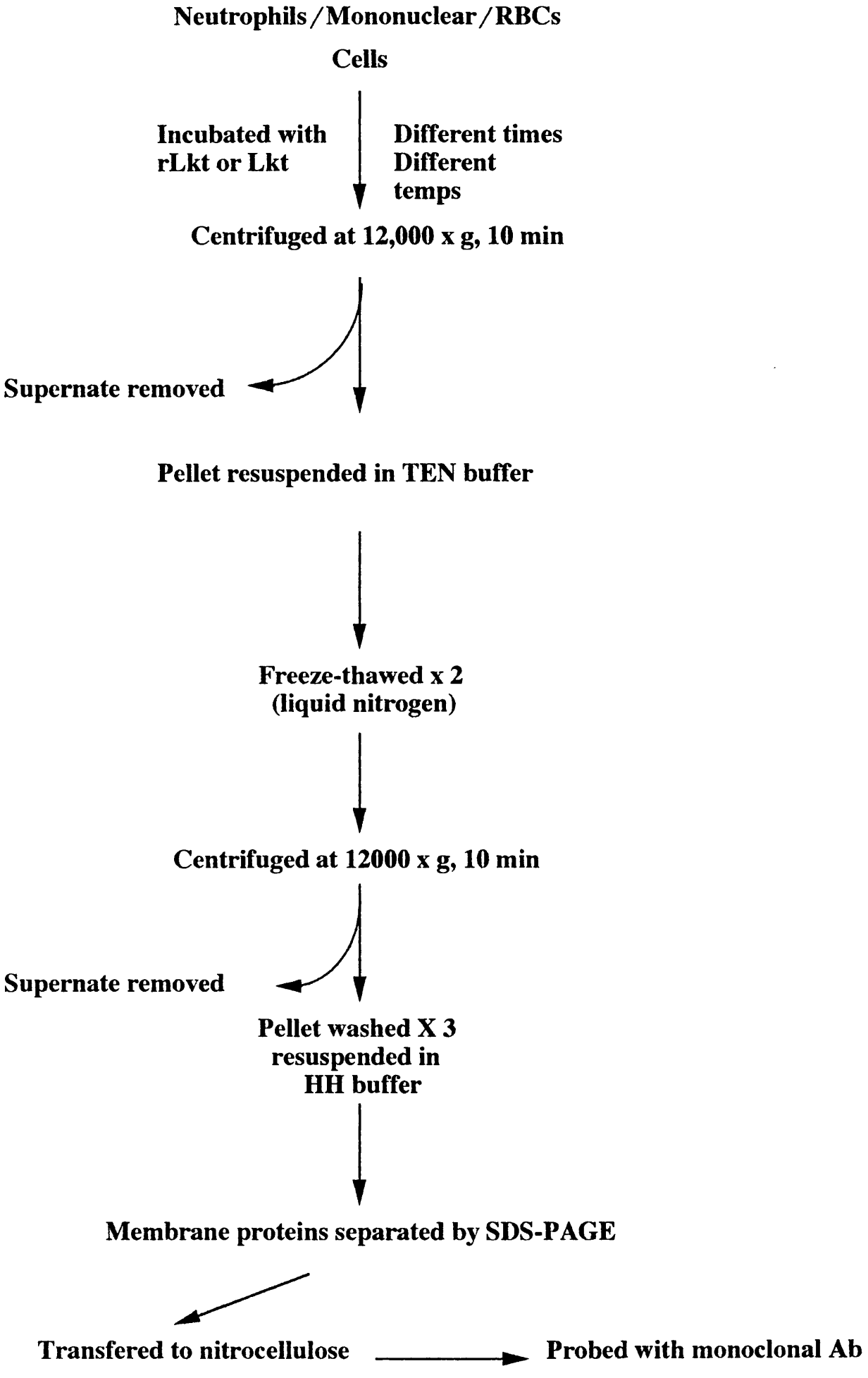
2.8 BINDING ASSAY

Purified preparations of RBCs, neutrophils, lymphocytes and monocytes cells, prepared as above (see sections 2.5.1 and 2.7) were resuspended in HH buffer containing 1 mM $CaCl_2$. RBCs (2×10^8 in 800 µl), neutrophils, lymphocytes or monocytes ($5 \times 10^{5)}$ were incubated at 37 °C for 1 h in a microfuge tube with 200 µl of culture supernate of *P. haemolytica* or with 50 µl of dialysed urea extracts containing rLktA from *E. coli*. The cells were separated by centrifugation at 15,000 x g for 15 min to removed unbound toxin and the supernates were used for ELISA. The pellets were resuspended in 1 ml TME buffer (containing 10 mM Tris/HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and freeze-thawed twice in liquid nitrogen. The membrane pellets were washed three times with TME buffer and finally resuspended in 100 µl of HH. The membrane proteins were separated by SDS-PAGE and then transferred to nitrocellulose by immunoblotting. An outline of the binding assay is shown in fig. 8. The blots were probed with monoclonal antibody against LktA or against AC toxin (9D4), and anti-mouse HRP conjugate (SAPU) (Scottish Antibody production Unit, Carluke) (see section 2.4).

2.9 ELISA

The wells of flat-bottomed, microtitre plates (DYNATECH IMMDULON$^{T.M}$, Chantilly, Virginia, USA) were coated with 350 µl of monoclonal antibody raised against rLktA and diluted 1 in 100 in 0.05 M carbonate buffer, pH 9.6 (appendix 4). The plates were incubated at 4 °C overnight in a humidified box. They were washed three times by carefully flooding with washing buffer, containing 0.5% gelatin, 0.01% Tween 20 in 100 ml PBS pH 7.4 and with each washing, the plates were inverted and dumped onto absorbent tissue to remove any remaining buffer from the wells. Native or rLktA was diluted in washing buffer and 300 µl volumes added to

Fig. 8. Binding assay



the wells. The plates were incubated for 2 h at 37 °C in a humidified box and then washed and dried as above. To each well, 250 µl rabbit polyclonal antibody raised against rLktA and diluted 1 in 3,000 in washing buffer was added. The plates were incubated for 4 h at 37 °C in a humidified box and then washed. 200 µl of a 1 in 3000 dilution of anti-rabbit IgG-HRP-enzyme conjugate (SAPU) in washing buffer was added to each well and the plate incubated overnight at 4 °C and washed as described above. The enzyme reaction was initiated by the addition of 200 µl of O-phenylenediamine (34 mg/ml) and H_2O_2 (20 µl) in 100 ml of citrate-phosphate buffer, pH 5.0 (freshly prepared, appendix 4). The plate was incubated for 30 min at room temperature in the dark for colour development. The reaction was stopped by addition to each well of 50 µl of H_2SO_4 (12.5% v/v). Absorbance was measured at 492 nm in an Anthos 2001 Elisa reader. An outline of the ELISA procedure is shown in fig. 9.

For measuring the level of polyclonal antibodies raised in rabbits against rLktA all procedures were the same except that instead of dilutions of rLktA, a standard amount of rLktA was added to each well and serial dilutions of the rabbit antisera were tested.

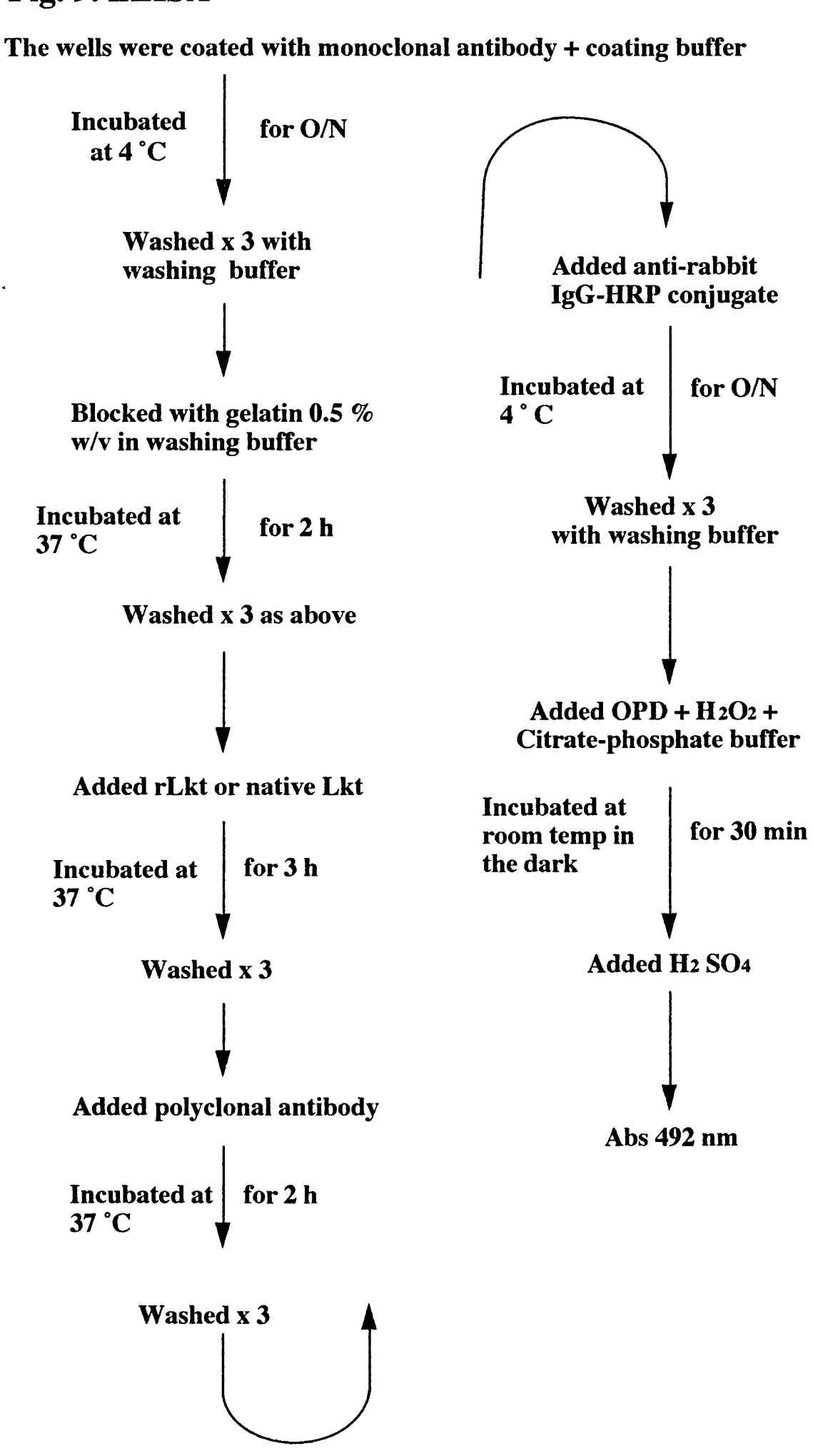
2.10 PROTEIN ESTIMATION

Protein estimation was carried out by the modified Lowry method of Markwell *et al.* (1978) using bovine serum albumin (Sigma) as a standard. The protein was measured at 610 nm using a spectrophotometer.

2.11 CHRISTIE ATKINS MUNCH-PETERSON (CAMP) TEST

This test detects co-haemolytic activity between ß-toxin produced by *Staphylococcus aureus* and other bacteria, and has been used as in the differentiation of *Streptococcus* species. Strains of *P. haemolytica* were

Fig. 9. ELISA


The wells were coated with monoclonal antibody + coating buffer
Incubated at 4 °C
for O/N
Washed x 3 with washing buffer
Blocked with gelatin 0.5 % w/v in washing buffer
Incubated at 37 °C
for 2 h
Washed x 3 as above
Added rLkt or native Lkt
Incubated at 37 °C
for 3 h
Washed x 3
Added polyclonal antibody
Incubated at 37 °C
for 2 h
Washed x 3
Added anti-rabbit IgG-HRP conjugate
Incubated at 4 ° C
for O/N
Washed x 3 with washing buffer
Added OPD + H2O2 + Citrate-phosphate buffer
Incubated at room temp in the dark
for 30 min
Added H2 SO4
Abs 492 nm

grown on BHIA containing 5% sheep blood for 18-24 h at 37 °C in air plus 10 % CO_2. *P. haemolytica* isolates were streaked at right angles, and in close proximity to *Staphylococcus aureus* which produces ß-toxin. A group B *Streptococcus* was used as positive control.

2.12 EFFECT OF LKT ON NEUTROPHIL CHEMOTAXIS

The micropore filter assay is a simple way of observing the reactions of neutrophils to chemotactic factors *in vitro*. It is essentially a two chamber apparatus in which neutrophils in the upper chamber are separated by a 5 µm filter from the chemotactic factor in the lower chamber.

To set up this assay, a rubber seal was first placed on top of the lower chamber. The chamber was then filled with 29 µl of chemotactic factor (OZ), or HH as a control, until a slight meniscus was seen. Care was taken to avoid trapping air bubbles. The shiny side of the filter was put on the top of the lower chamber and the upper chamber put in place and tightened by 6 screws. The apparatus was incubated for 10 min at 37 °C in 10 % CO_2. The neutrophils, with or without toxin, were then placed in the upper chamber and incubated for 45 min at 37 °C in 10 % CO_2. The chamber was then placed upside down and the two halves separated. The filter was removed and dried at room temperature. The dull side of the filter was washed in PBS and a rubber policeman was used to scrape off excess moisture. The filter was fixed in methanol for 2 min and stained with neat Leishman's stains (BDH Ltd) for 6 min and then with 50% Leishman's stains for 6 min. The excess stain was removed by washing with tap water. The filter was then mounted with DPX on a chemotaxis slide and the migrated cells were counted under the microscope with a 40 x objective. Five fields per well were counted and the average taken. In some experiments, the LktA preparations themselves were tested for their chemotactic activity by adding dilutions to the lower chamber

in place of OZ.

2.13 EFFECT OF LKT ON LEUKOCYTE MORPHOLOGY AND MOVEMENT

Human and bovine neutrophils were contained in a filming chamber: a stainless steel slide 70 x 40 x 1 mm with a 15 mm-diameter circular aperture and a 22 x 22 mm square glass coverslip was fixed over the aperture with paraffin wax. One ml of 0.5% BSA was added into the well. After 1 h, the solution of BSA was discarded and 200 µl of cell suspension, 1 x 10^6 /ml in HH, was added to the well and left for 15 min at room temperature. The non-adherent cells were removed by washing the well with HH. The adherent cells were treated with a 300 µl dilution of the active or inactive form of rLktA from *E. coli* or native leukotoxin from *P. haemolytica*. The top of the well was sealed with hot paraffin. The chamber was placed on the stage of an inverted microscope within a temperature-controlled (37 °C) box and locomotion was observed by means of a video camera connected to a monochrome monitor and also to an Archimedes A 5000 computer with a Watford video digitiser programmed to capture and analyse one frame every 5 seconds. The tracking program selected up to 80 phase bright cells. After tracking for 2 min, the computer displayed results which showed persistence (P), diffusion coefficient (D) and speed (S) of neutrophil locomotion which can be determined from the equation $D=S^2 (1+P)$. The speed of neutrophils was observed by displacement of randomly moving objects determined by the least squares approximation in a measured time interval and gives an apparent speed that approaches the true speed as the measuring interval decreases. Drift (a component of non-random locomotion) was calculated by determining the centre of cell density by carrying out a vectoral sum of the X and Y displacements of each cell centre to determine the average population displacement.

2.14 ACTIVATION OF LKT *IN VITRO*

The non-activated recombinant LktA (rLktA) was activated by addition of LktC and cytosolic activating factor (CAF), an *E. coli* cell extract, to LktA *in vitro*. LktA was prepared from *E. coli* strain SY327λ pir containing plasmid pGW64 (encoding *lktA*) and the LktC was prepared from *E. coli* strain SY327λ pir containing plasmid pGW78 (encoding *lktC*). Cytosolic activating factor was prepared from *E. coli* strain SY327λ pir at 37 °C. Cultures were grown with shaking, at OD 0.6, they were centrifuged at 15,000 x g for 15 min at 4 °C and the cells were sonicated. The resulting supernate was separated from the pellet by centrifugation and used as CAF. For activation of rLktA, 50 µl of urea extract containing rLktA was added to mixture of 50 µl of urea extract containing rLktC and 50 µl of CAF and incubated for 30 min at 4 °C. The mixture was then added to bovine, human or rabbit neutrophils and incubated for a further 20 min at 38 °C. The cells were then tested in the CL-inhibition assay (section 2.5). These experiments were done in collaboration with E. K. Hormozi and Dr. G. Westrop

3. RESULTS

3.1 CHARACTERISATION OF BACTERIA

The API 20 NE (see appendix 1) was used to confirm the identity of some of the *P. haemolytica* isolates which were commonly used in this study. All other strains used had been identified and serotyped by Ali *et al.* (1992); Azad *et al.* (1992); Davies *et al.* (1992) or Donachie (personal communication) and preserved as frozen stocks or freeze-dried. As appendix 2 shows, most of the tests were negative. However, the typical positive and consistent reactions of *P. haemolytica* were found in the reduction of nitrates to nitrites, production of ß-galactosidase (majority of strains) and oxidase. Indole production was found in one *P. haemolytica* isolate (Ph6), and in *P. multocida* (both strains). *Pseudomonas aeruginosa* NCTC 6749 was used as a control organism for the API test and gave the expected biochemical profile. The serotypes of the strains used most frequently throughout this study were checked by the indirect haemagglutination method (section 2.1.2). The above results show that the strains to be used in the present study were confirmed as *P. haemolytica* except possibly Ph6, which was atypical in other characteristics but was serotype A1 (Ali *et al.*, 1992) and possessed one plasmid in common with *P. haemolytica* (Azad *et al.*, 1992).

3.2 PRODUCTION OF LEUKOTOXIN

3.2.1 Cultural conditions affecting production of native leukotoxin

Thirty three *P. haemolytica* isolates, including representatives of all different serotypes and untypables, were examined for Lkt production in culture supernates. For detection and quantitation of LktA, different methods were used including SDS-PAGE (7.5%, 10% and 12.5% acrylamide gels) and immunoblotting with monoclonal antibody, rabbit polyclonal antibody or

bovine convalescent serum, chemiluminescence (CL) inhibition assay, ELISA chemotaxis and cell tracking assay.

3.2.1.1 Age of culture

The age of the culture was found to have a major influence on LktA yield. For example, strain Ph2 was grown in 50 ml volumes of BHIB at 37 °C in a 250 ml shake flask and culture supernate samples were removed after 2, 3, 4, 5, 6, 8 and 12 h. The immunoblot profiles of these culture supernates developed with a rabbit polyclonal antibody raised against recombinant LktA are shown in fig. 10a. The leukotoxin antigen was seen as a prominent band at 105 kDa. Some faint, lower mol. wt. bands were apparent in some samples. The time of maximum production of LktA for isolate Ph2 was around 6 h, as judged by immunoblotting and as measured by the CL inhibition assay (figs. 10a-b) and toxin production with peak production of the end of log phase (6 h). The same result was found with two other strains (Ph12, fig. 10c; Ph144, fig. 10d). Thus, with these strains, there was good agreement between the amount of LktA antigen produced in the culture supernate and the amount of LktA activity detected. These results indicate that production of LktA by those strains in BHIB was dependent on active growth of bacteria. The timing of the end of log phase varied somewhat from strain to strain (table 4). As the culture then aged, there was a marked decline in toxin activity (figs 10b; 10c; 10d). Immunoblot profiles of culture supernates of Ph2, Ph8, Ph10, Ph30, Ph42, Ph44 and Ph72 after growth for 24 h in BHIB show two or three bands which cross reacted with an anti-LktA monoclonal antibody, thus indicating degradation of the toxin (fig. 11b). The immunoblot profiles of these culture supernates were also developed with convalescent serum (fig. 11a).

Fig. 10.

Production of leukotoxin in the culture supernate.

P. haemolytica Ph2 was grown in 50 ml BHIB in 250 ml shake flask at 120 rpm at 37 °C and samples were removed at intervals. The cell-free culture supernate/samples were resolved by SDS-PAGE in a 10% polyacrylamide gel, immunoblotted and probed with rabbit polyclonal antibody raised against rLktA at[a] dilution of 1 in 2000. Arrow shows bands (105 kDa) identified as LktA.

a

Lane 1: culture supernate harvested at 2 h

Lane 2: culture supernate harvested at 3 h

Lane 3: culture supernate harvested at 4 h

Lane 4: culture supernate harvested at 5 h

Lane 5: culture supernate harvested at 6 h

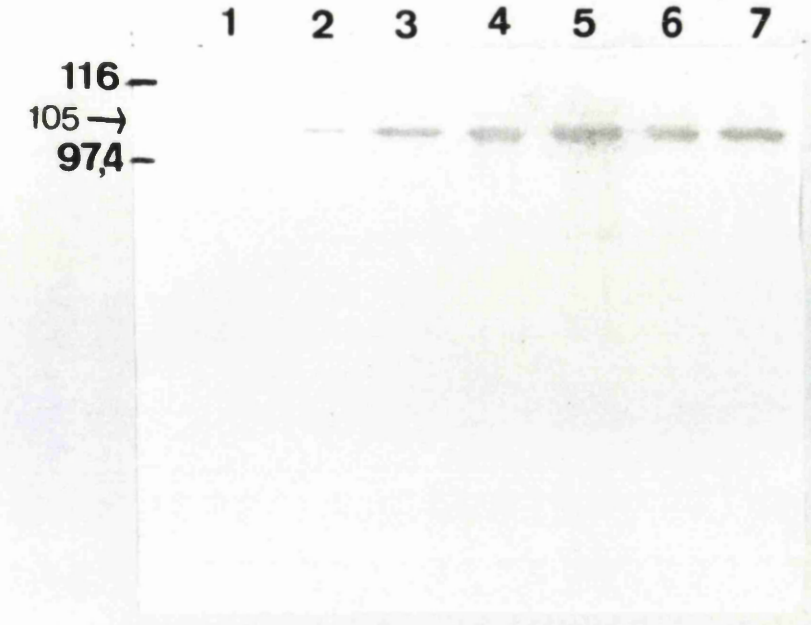
Lane 6: culture supernate harvested at 8 h

Lane 7 : culture supernate harvested at 12 h

b

Growth of *P. haemolytica* Ph2 (OD 620 nm) and toxin activity in culture supernate (CL-inhibition assay with bovine neutrophils).

a


1
2
3
4
5
6
7
116
105
97,4

(b)

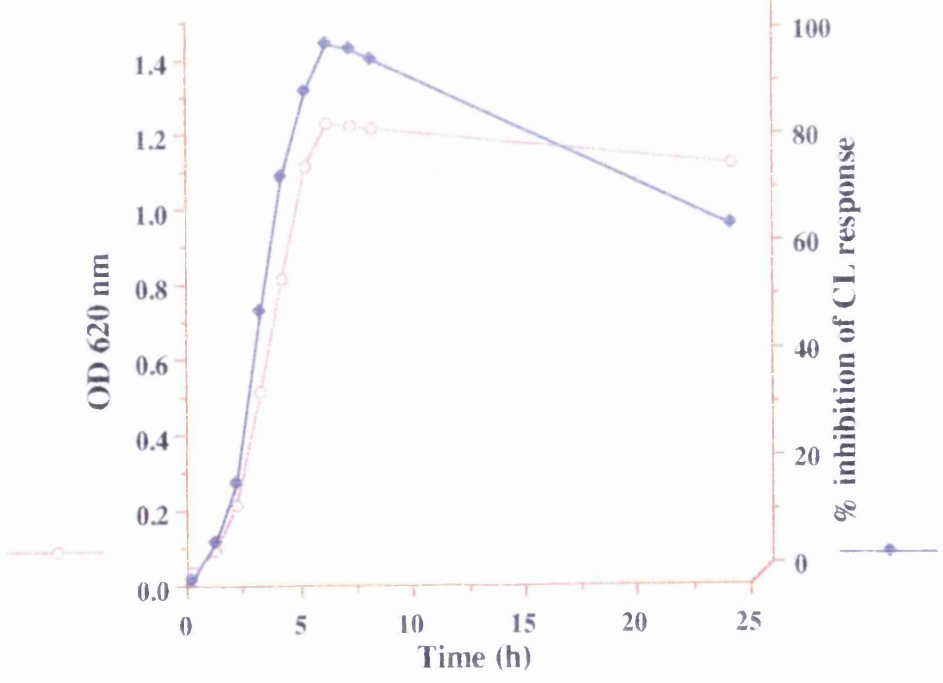

OD 620 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time (h)
100
80
60
40
20
0
% inhibition of CL response

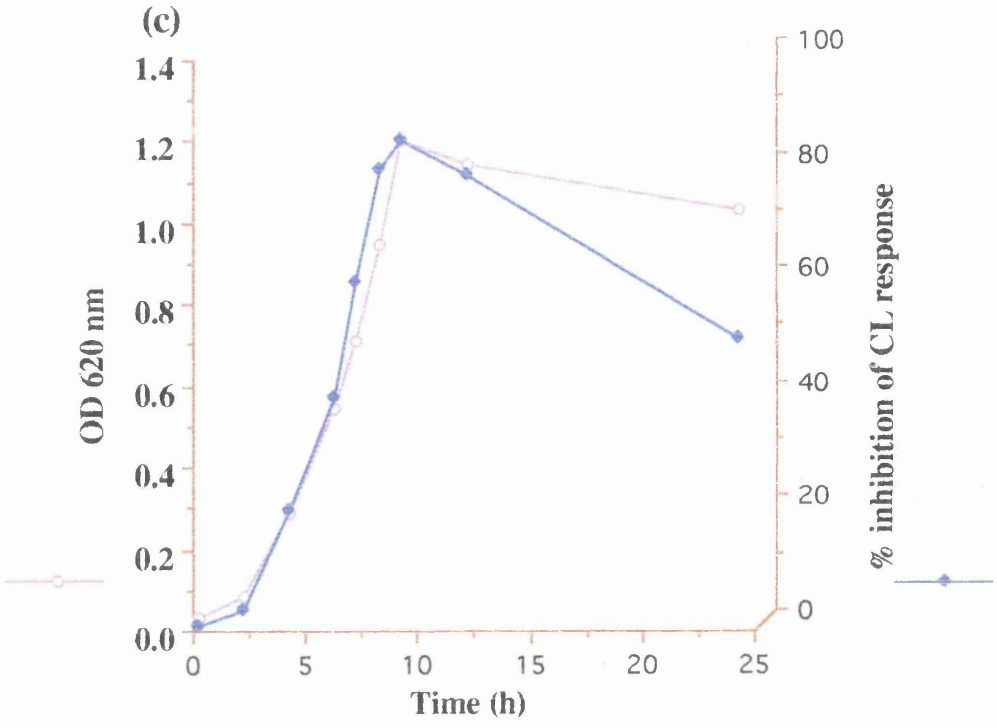
Fig. 10.

c

Growth of *P. haemolytica* Ph12 (OD 620 nm) and toxin activity in culture supernate (CL-inhibition assay with bovine neutrophils).

d

Growth of *P. haemolytica* Ph144 (OD 620 nm) and toxin activity in culture supernate (CL-inhibition assay with bovine neutrophils)


(c)
OD 620 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time (h)
100
80
60
40
20
0
% inhibition of CL response

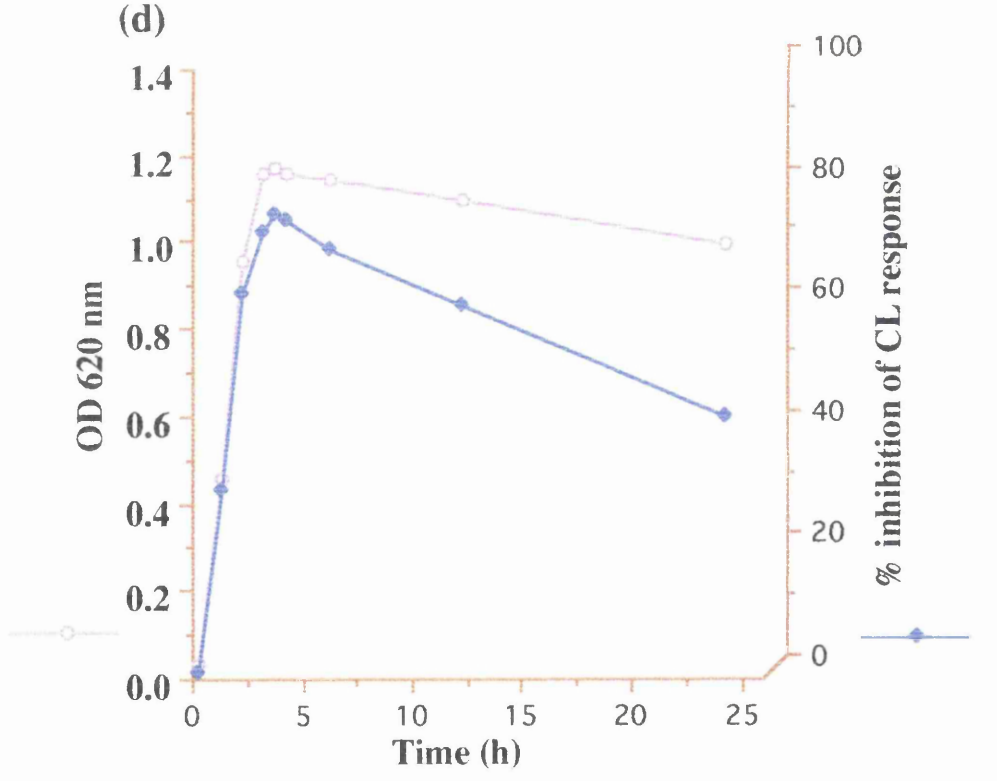

(d)
OD 620 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time (h)
100
80
60
40
20
0
% inhibition of CL response

Fig. 11.

Antigenic relationships between leukotoxin proteins of *P. haemolytica* isolates.

Cell-free culture supernate of *P. haemolytica* isolates were removed at the end of log phase (e.g. 6 h) (lanes 1-7) and at 24 h (lanes 8-14) and were resolved by SDS-PAGE (10% polyacrylamide gel) and immunoblotted and probed with convalescent serum raised against Ph2 at a dilution of 1 in 200 (a) or with monoclonal antibody raised against of rLktA (b). Arrows show bands identified as LktA.

Culture supernate was taken at the end of log phase growth of bacteria

Lane 1: Ph2

Lane 2: Ph8

Lane 3: Ph10

Lane 4: Ph30

Lane 5: Ph42

Lane 6: Ph44

Lane 7: Ph72

Culture supernate was taken after 24 h growth of bacteria

Lane 8: Ph2

Lane 9: Ph8

Lane 10: Ph10

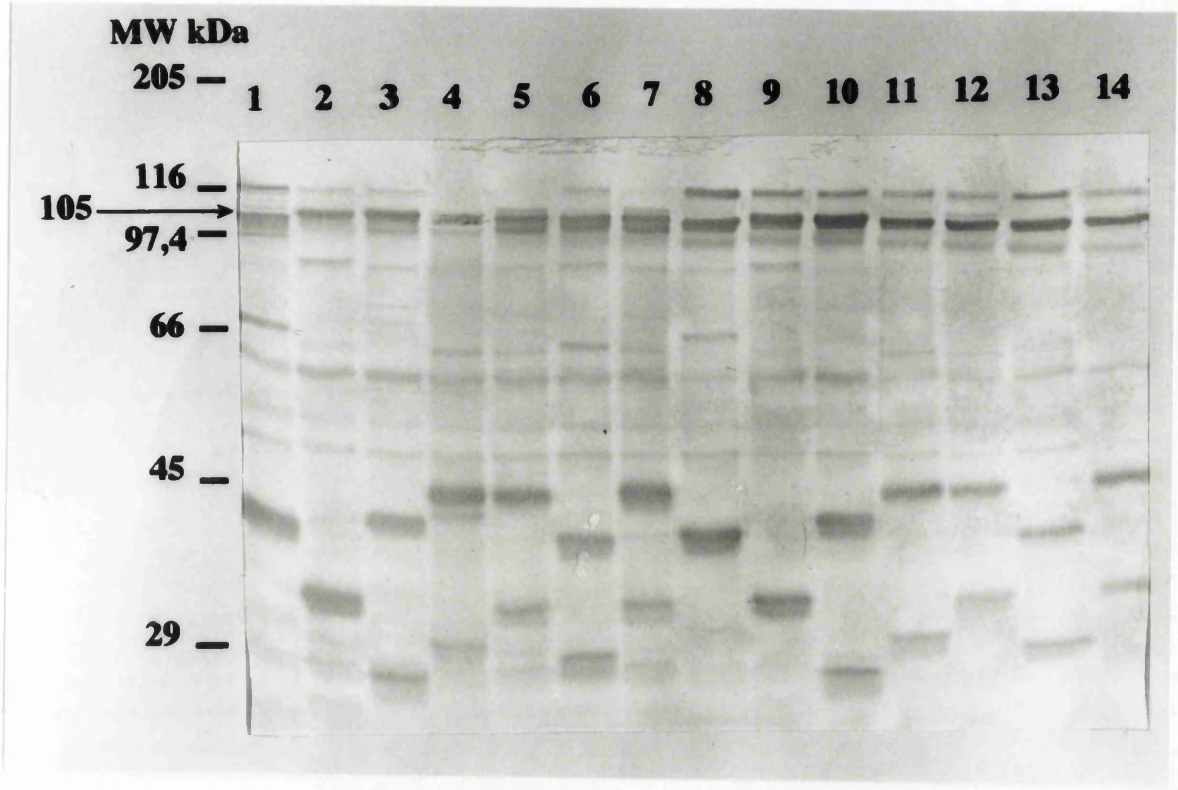
Lane 11: Ph30

Lane 12: Ph42

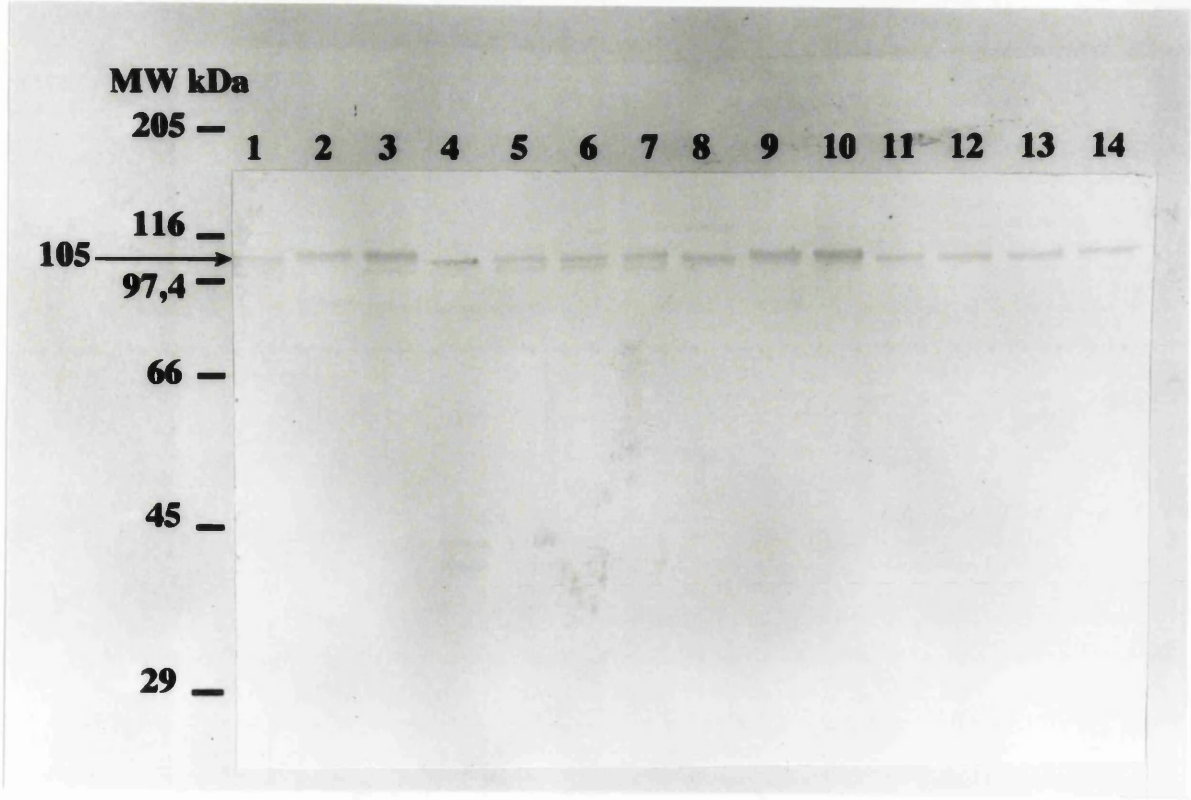
Lane 13: Ph44

Lane 14: Ph72

a


MW kDa
205
116
105
97,4
66
45
29
1 2 3 4 5 6 7 8 9 10 11 12 13 14

b


MW kDa
205
116
105
97,4
66
45
29
1 2 3 4 5 6 7 8 9 10 11 12 13 14

3.2.1.2 Aeration conditions

In further experiments, growth of the *P. haemolytica* Ph2 isolate under various conditions of aeration was monitored by spectrophotometry and LktA production and toxic activity was measured by immunoblotting and CL assays. Various conditions of aeration of the culture, i.e. very high aeration, high aeration, moderate aeration, low aeration, no aeration and anaerobic (5% CO_2), according to Davies *et al.* (1992) were examined. The maximum yield of toxin and growth was obtained with very high aeration i.e. with 50 ml of culture medium contained in a 250 ml dimpled shake flask and incubated at 120 rpm and 37 °C (fig. 12a-c). As figs 12a and 12c show, LktA production was dependent on growth of the bacteria. Maximum growth occurred with very high and high aeration (fig. 12a). Fig. 12c shows the coomassie blue stained gel of culture supernate samples and the band at 105 kDa (arrow shows band presumed to be leukotoxin). Other protein material was present in samples taken at 24 h. Production of LktA (fig. 12d) as judged by immunoblotting of the culture supernate samples with anti-LktA polyclonal was greatest with very high and high aeration (lane 5 and 6) and reduced with moderate aeration (lane 4), low aeration (lane 3) and anaerobic (5% CO_2) (lane 2). No LktA was detected by this method when bacteria were grown without shaking (figs 12c and 12d lane 6 and 1, respectively). When LktA activity of these same samples was monitored by CL assay, similar results were obtained. The bacterium produced most LktA activity with very high and high aeration (fig. 12b).

3.2.1.3 Growth temperature

In other experiments *P. haemolytica* Ph2 was grown in BHIB for 5 h at 37 °C and then for 1 h at different temperatures. Each culture was then centrifuged at 7,000 x g for 15 min and culture supernate resolved by 10%

Fig. 12.

Effect of aeration conditions on growth of *P. haemolytica* strain Ph2 and leukotoxin production.

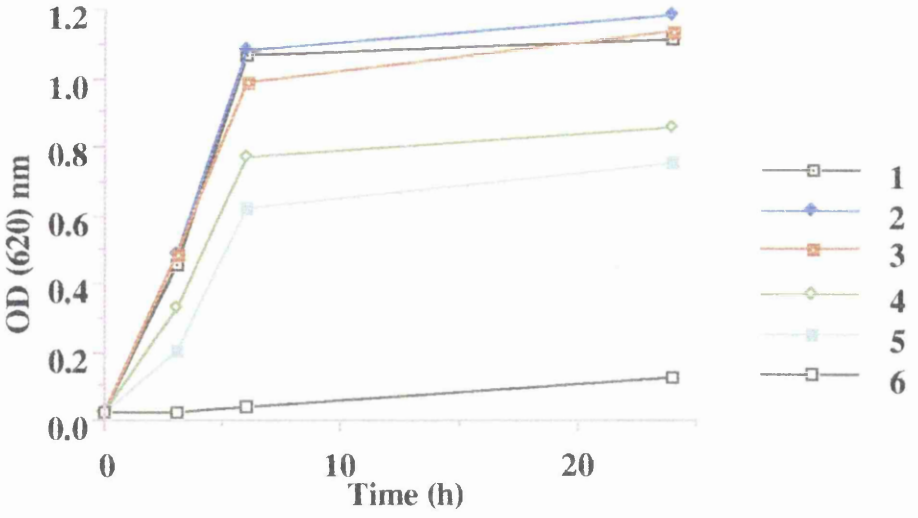
Strain Ph2 was grown in 50 ml of BHIB at 37 °C under various conditions i.e. very high aeration, high aeration, moderate aeration, low aeration, anaerobic + 5% CO_2, without shaking and the OD_{620} was recorded at intervals (a). The LktA activity of the culture supernate samples was measured by the CL inhibition assay. Culture supernate samples were incubated with bovine neutrophils for 30 min at 38 °C, then CL responses of the neutrophils were measured after stimulation with OZ. The data presented are the means of the peak CL responses during the 45 min test period after stimulation (b). Cell-free culture supernate/samples of each culture were removed at the end of log, phase (6 h) and at 24 h and were resolved by SDS-PAGE (10% polyacrylamide gel) and stained with coomassie blue (c), or immunoblotted and probed with polyclonal antibody raised against rLktA at a dilution of 1 in 2000 (d). Arrows show bands identified as LktA.

Key to fig: 12 a 6 h growth of bacteria

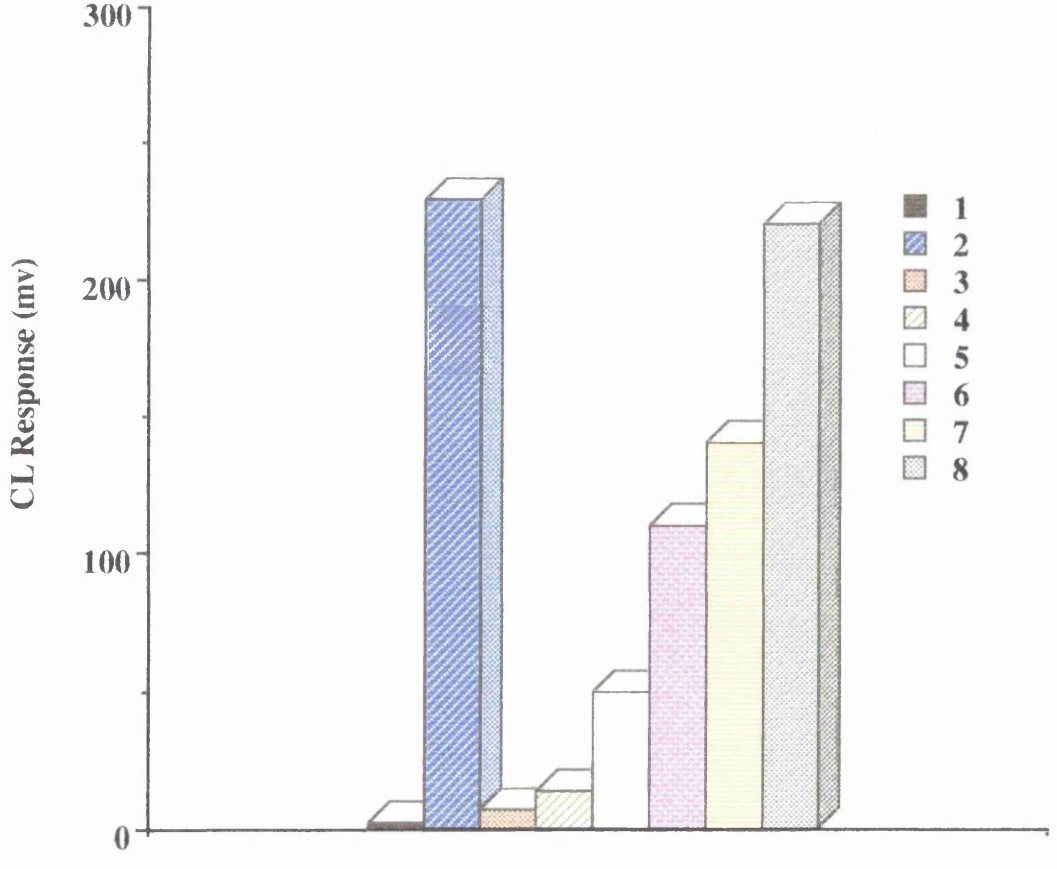
1:	Very high aeration
2:	High aeration
3:	Moderate aeration
4:	Low aeration
5:	Anaerobic + 5% CO_2
6:	Without shaking

1:	Control (BHIB)
2:	Control (BHIB + OZ)
3:	Very high aeration
4:	High aeration
5:	Moderate aeration
6:	Low aeration
7:	Anaerobic + 5% CO_2
8:	Without shaking

(a)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (620) nm
0
10
20
Time (h)
1
2
3
4
5
6

(b)


300
200
100
0
CL Response (mv)
1
2
3
4
5
6
7
8

Keys to fig 12c and 12d

(c)

1: Mol. wt marker SDS 6H

2: Very high aeration

3: High aeration

4: Moderate aeration

5: Low aeration

6: Anaerobic + 5% CO_2

7: Without shaking

(d)

1: Without shaking

2: Anaerobic + 5% CO_2

3: Low aeration

4: Moderate aeration

5: High aeration

6: Very high aeration

Lanes 8-13 in fig 12 (c) are for samples taken after 24 h growth of the bacteria

8: Very high aeration

9: High aeration

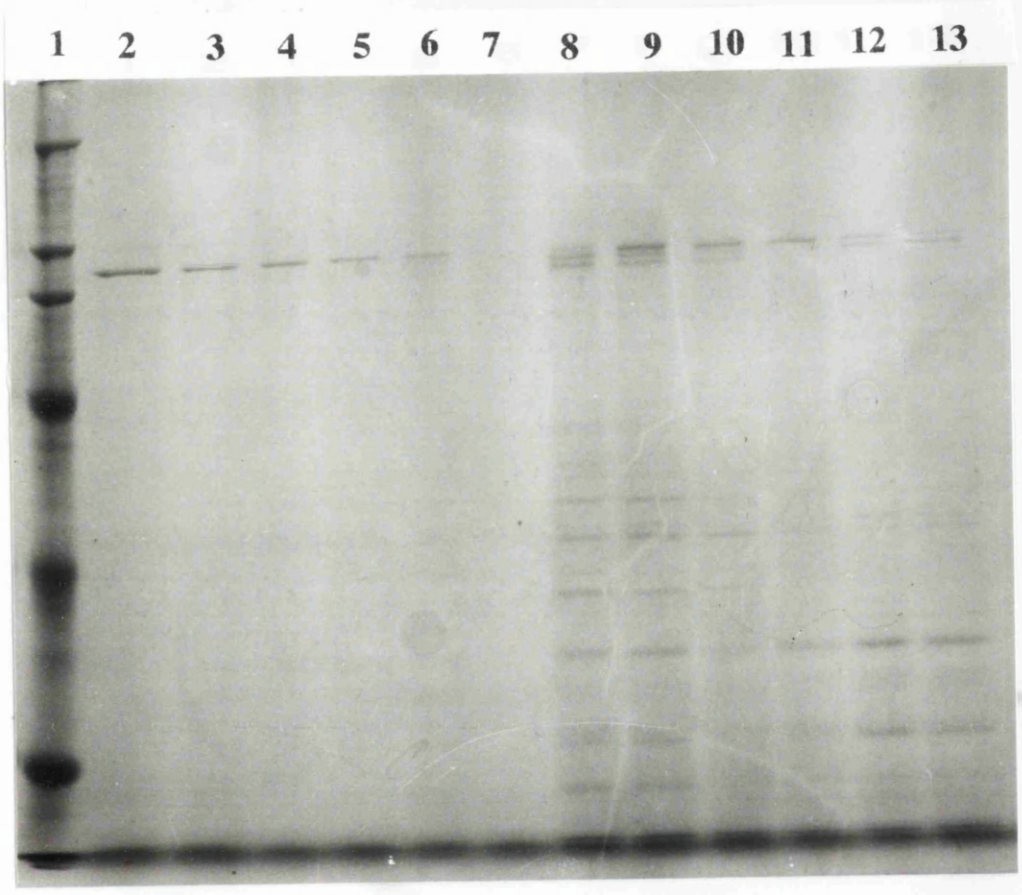
10: Moderate aeration

11: Low aeration

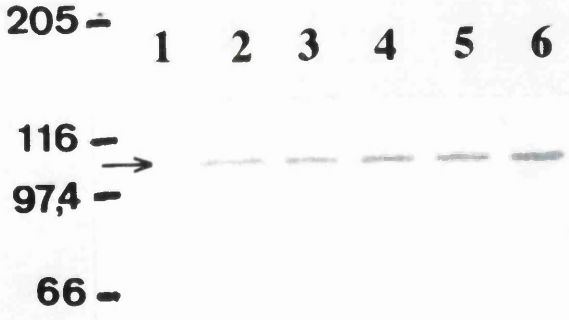
12: Anaerobic + 5% CO_2

13: Without shaking

c



d



SDS-PAGE and transferred to nitrocellulose and developed with convalescent serum. Fig. 13 shows the production of LktA at the different temperatures. Lanes 1, 2, 3, 4, 5, 6 are culture supernates of Ph2 when bacteria were grown at 41, 37, 35, 30, 25 and 20 °C, respectively. In this immunoblot, convalescent serum was used to detect LktA (before the polyclonal serum was available) and, as shown in fig. 13, bands in addition to the 105 kDa LktA antigen were detected. *P. haemolytica* produced more toxin and grew optimally when grown at 37 °C (data not shown). The production of LktA was thus shown to be temperature dependent and with increasing temperature, the production of LktA was increased. However the production of LktA decreased when bacteria were grown at 41 °C (fig. 13 lane 1). A faint band of LktA was detected when bacteria were grown at 20 °C (fig. 13 lane 6).

3.2.1.4 Growth medium

The production of some bacterial toxins is known to be regulated by the composition of the growth medium.

Calcium and iron. To show the effect of calcium and iron on production and secretion of LktA, *P. haemolytica* Ph2 was grown in the presence of different concentrations of iron ($FeCl_3$ 250 μM, 500 μM, 1 mM, 2 mM), 2-2'dipyridyl (50 mM, 100 mM, 200 mM), calcium ($CaCl_2$ 50 mM, 100 mM, 200 mM) and EGTA (8 mM, 16 mM, 32 mM) in BHIB. Strain Ph2 was grown initially in BHIB for 5 h at 37 °C. The culture was then centrifuged at 7,000 x g for 15 min and the pellet suspended in BHIB containing either iron, calcium, 2-2'dipyridyl or EGTA. The bacteria were grown for a further 1 h at 37 °C and culture supernates taken and LktA production assayed by SDS-PAGE and immunoblotting. The growth of bacteria over a 1 h period was

Fig. 13.

Effect of temperature on LktA prodction by *P. haemolytica* Ph2.

P. haemolytica Ph2 was grown at various temperatures in 50 ml BHIB in a 250 ml shake flask at 120 rpm at 37 °C. The culture supernate was taken at 6 h and resolved by SDS-PAGE and immunoblotted and developed with bovine convalescent serum. Arrow shows bands at 105 kDa identified as LktA.

Lane 1: growth at 41 °C
Lane 2: growth at 37 °C
Lane 3: growth at 35 °C
Lane 4: growth at 30 °C
Lane 5: growth at 25 °C
Lane 6: growth at 20 °C

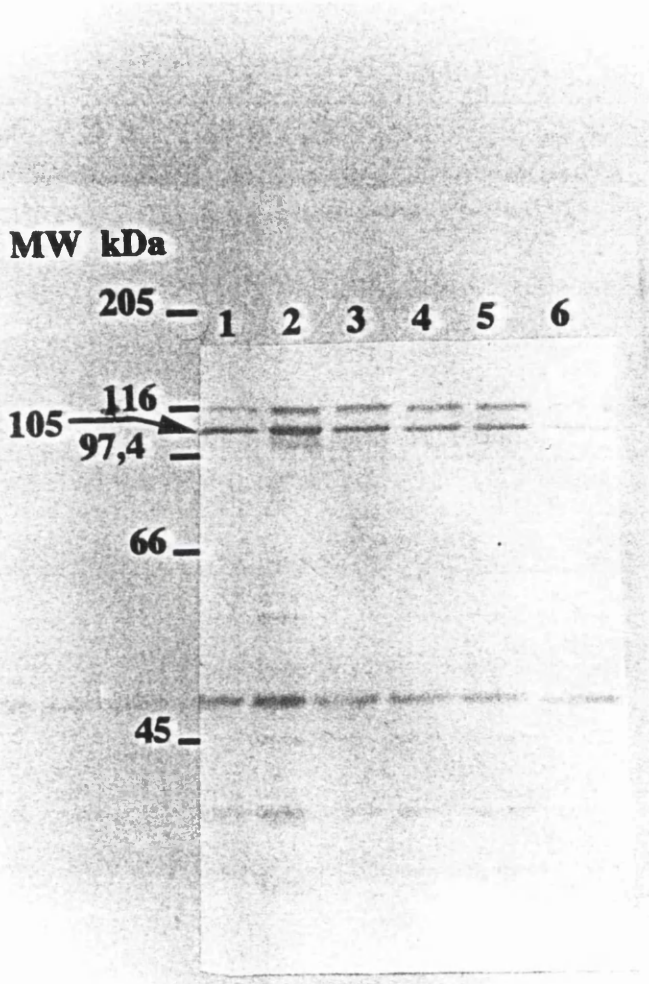

MW kDa
205
116
105
97,4
66
45
1
2
3
4
5
6

Fig. 14.

Effect of calcium and iron on growth of *P. haemolytica* strain Ph2 and production and secretion of LktA.

P. haemolytica Ph2 was grown in 50 ml BHIB in a 250 ml shake flask at 120 rpm for 5 h at 37 °C. The culture was centrifuged at 7000 x g for 15 min and the pellet suspended in BHIB containing either iron, calcium, dipyridyl or EGTA at various concentration.Incubation was continued for a further 1 h at 37 °C. Growth was monitored spectrophotometricaly at OD_{620} nm (a). Cell free culture supernate was taken at 6 h and resolved by SDS-PAGE and immunoblotted and probed with bovine convalescent serum (b).

a

1: growth in BHIB (control)

2: growth in BHIB + 8 mM EGTA

3: growth in BHIB + 16 mM EGTA

4: growth in BHIB + 32 mM EGTA

5: growth in BHIB + 50 mM dipyridyl

6: growth in BHIB + 100 mM dipyridyl

7: growth in BHIB + 200 mM dipyridyl

8: growth in BHIB + 50 mM $CaCl_2$

9: growth in BHIB + 100 mM $CaCl_2$

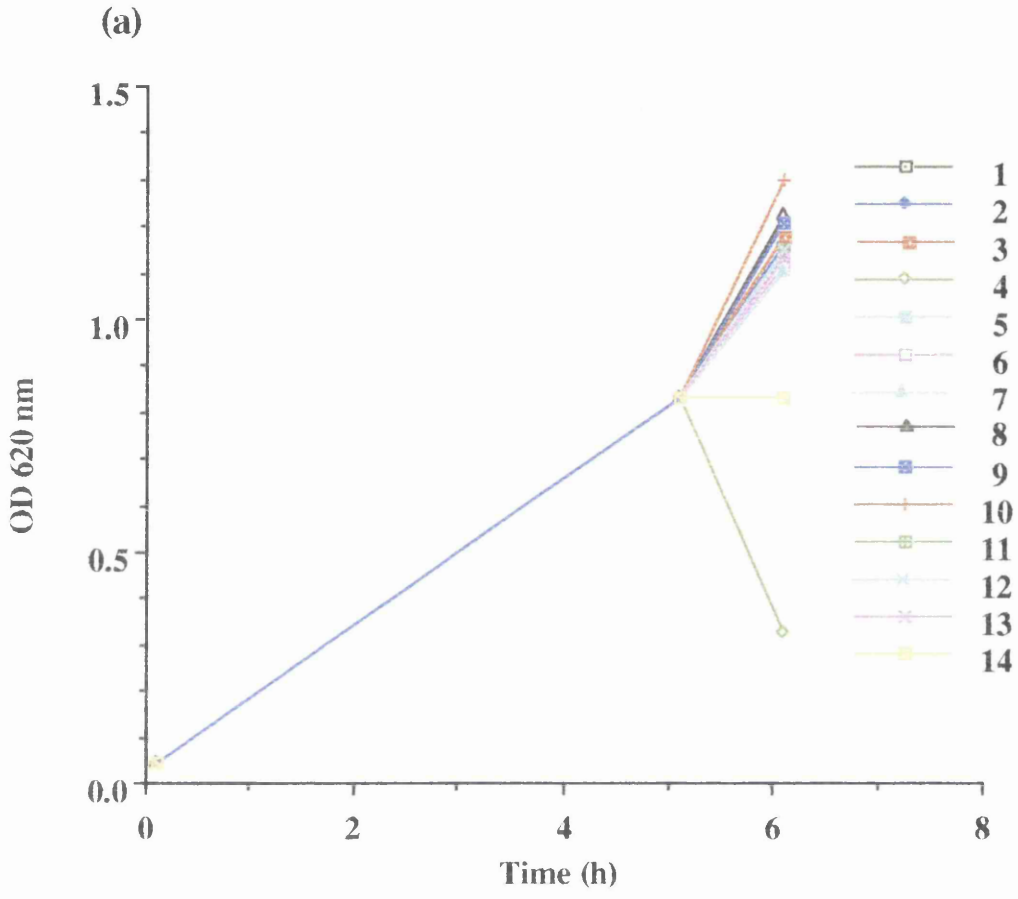
10: growth in BHIB + 200 mM $CaCl_2$

11: growth in BHIB + 250 µM $FeCl_3$

12: growth in BHIB + 500 µM $FeCl_3$

13: growth in BHIB + 1 mM $FeCl_3$

14: growth in BHIB + 2 mM $FeCl_3$


(a)
1.5
1.0
0.5
0.0
OD 620 nm
0
2
4
6
8
Time (h)
1
2
3
4
5
6
7
8
9
10
11
12
13
14

b

1: Culture supernate from BHIB medium contanining 8 mM EGTA

2: Culture supernate from BHIB medium contanining 16 mM EGTA

3: Culture supernate from BHIB medium contanining 100 mM dipyridyl

4: Culture supernate from BHIB medium contanining 200 mM dipyridyl

5: Culture supernate from BHIB medium (control)

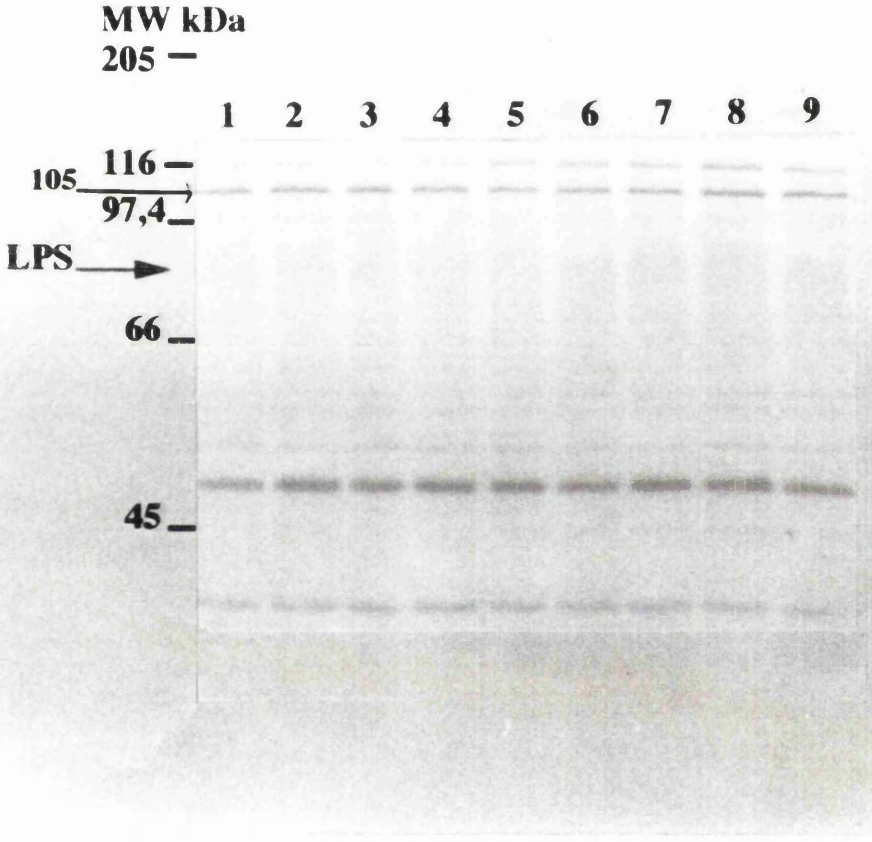
6: Culture supernate from BHIB medium contanining 100 mM $CaCl_2$

7: Culture supernate from BHIB medium contanining 200 mM $CaCl_2$

8: Culture supernate from BHIB medium contanining 500 μM $FeCl_3$

9: Culture supernate from BHIB medium contanining 1 mM $FeCl_3$

b



inhibited when the bacteria were grown in the presence of the highest concentrations of iron (2 mM) and EGTA (32 mM) (fig. 14a). Fig. 14b shows the production of LktA was not influenced by the presence of 1 mM iron (lane 9), 200 mM calcium (lane 7), 200 mM 2-2'dipyridyl (lane 4) and 16 mM EGTA (lane 2) i.e. concentrations which did not significantly inhibit growth of the organism. These results indicate that the amount of iron and calcium was not critical for production and secretion of LktA in BHIB, at least over a 1 h period.

Osmolarity. Alterations in DNA supercoiling created by changes in osmolarity are known to effect the regulation of expression of several bacterial proteins (Carmona *et al.*, 1993). Different concentrations of NaCl in BHIB were tested to determine the effect of osmolarity on LktA production. *P. haemolytica* Ph2 was grown in BHIB as above and after 5 h the bacteria were suspended in BHIB containing different concentrations of NaCl for 1 h. Fig. 15b shows an immunoblot of the resulting culture supernate and fig. 15a shows the growth of the bacteria in the different concentrations of NaCl. The bacteria produced LktA normally when grown in 0.1, 0.3 and 0.5 M NaCl (fig. 15b lanes 1, 2, 3). However, the bacteria did not produce detectable LktA in the presence of 0.8, 1 or 1.2 M NaCl (fig. 15b lanes 4, 5, 6). The highest concentrations (≥ 1.0 M) prevented growth of the organism (fig. 15a), but there was only slight retardation of growth of the bacteria in lower salt concentrations (fig. 15a) particularly at 0.8 M where little or no LktA was detected. In this immunoblot, convalescent serum was used to detect LktA and the antibody detected other products of *P. haemolytica* in the culture supernate. The arrow shows the band presumed to be leukotoxin.

Different concentrations of glucose and sucrose were also investigated. *P. haemolytica* Ph2 was grown as previously described, and after 5 h, the

Fig. 15.

Effect of NaCl or glucose on growth of *P. haemolytica* Ph2 and production of LktA.

P. haemolytica Ph2 was grown in 50 ml volumes of BHIB for 5 h at 37 °C. The culture were then centrifuged at 7000 x g for 15 min and the pellet suspended in 50 ml volumes of BHIB containing different concentration of NaCl or glucose for 1 h. Growth was monitored by spectrophotometry at 620nm (a). Cell-free culture supernates were taken and resolved by SDS-PAGE, immunoblotted and probed with bovine convalescent serum. Arrow shows the band at 105 kDa presumed to be LktA (b).

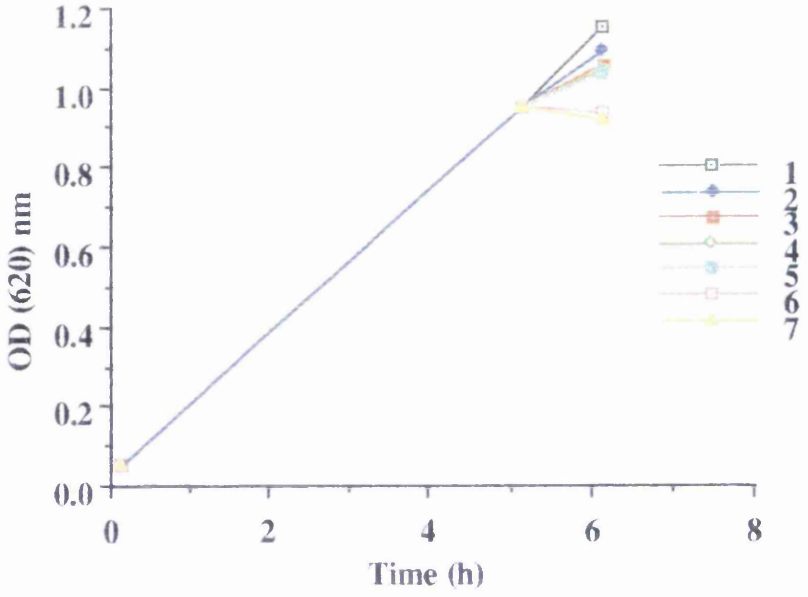
a

1: BHIB control
2: BHIB + 0.1 M NaCl
3: BHIB + 0.3 M NaCl
4: BHIB + 0.5 M NaCl
5: BHIB + 0.8 M NaCl
6: BHIB + 1 M NaCl
7: BHIB + 1.2 M NaCl

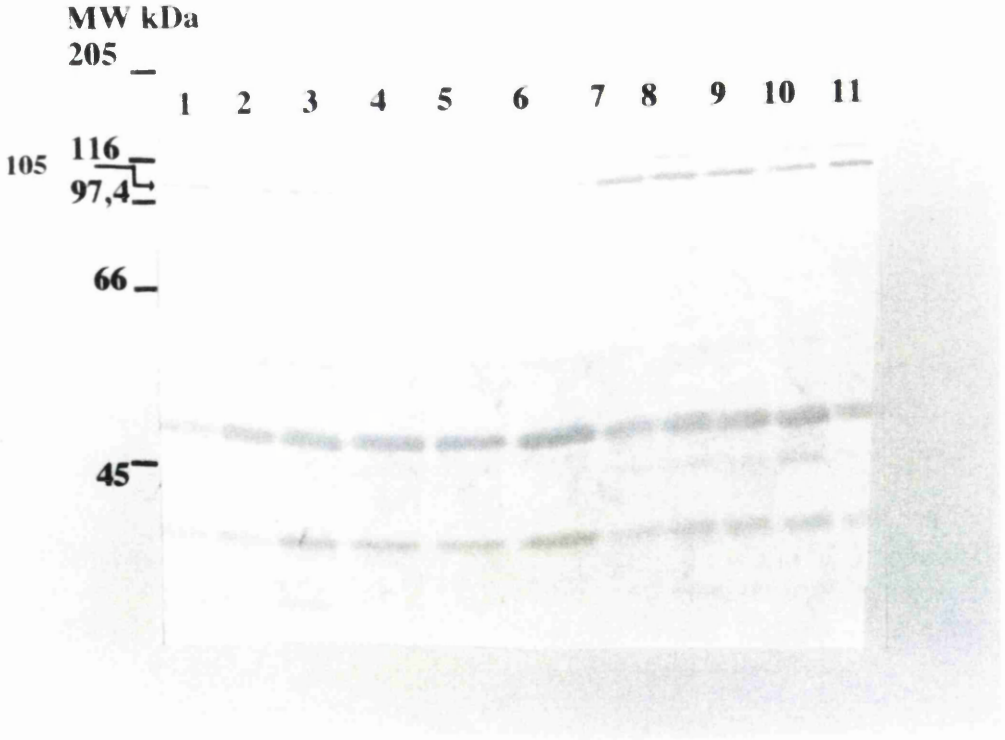
b

Lane 1: BHIB + 0.1 M NaCl
Lane 2: BHIB + 0.3 M NaCl
Lane 3: BHIB + 0.5 M NaCl
Lane 4: BHIB + 0.8 M NaCl
Lane 5: BHIB + 1 M NaCl
Lane 6: BHIB + 1.2 M NaCl
Lane 7: BHIB + 0.1 M glucose
Lane 8: BHIB + 0.3 M glucose
Lane 9: BHIB + 0.7 M glucose
Lane 10: BHIB + 1 M glucose
Lane 11: BHIB control

(a)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (620) nm
0
2
4
6
8
Time (h)
1
2
3
4
5
6
7

b


MW kDa
205
1
2
3
4
5
6
7
8
9
10
11
105
116
97,4
66
45

growing bacteria were resuspended in BHIB containing different concentrations of glucose and sucrose. After the culture supernates were taken and LktA production assayed by SDS-PAGE and transferred to nitrocellulose and developed with convalescent serum. Figs. 15b and 16 show the production of LktA by Ph2 in BHIB containing glucose and sucrose. Strain Ph2 produced LktA normally when it was grown in BHIB containing glucose or sucrose of 0.1 M, 0.3 M, 0.7 M and 1 M. The production of LktA when grown in BHIB alone, is also shown. The results indicate that different concentration of glucose and sucrose had no effect on production of LktA.

pH. Production of LktA at different pH values was also examined. *P. haemolytica* Ph2 isolate was grown as above and, after 5 h the bacteria were resuspended in BHIB in which the pH had been changed by the addition of NaOH or HCl. *P. haemolytica* produced more toxin in alkaline (pH 8) medium than in acidic (pH 6) medium (fig. 16, lanes 6 and 7 respectively) but the amount in the former was comparable to that produced in normal BHIB (pH 7.3).

Antibiotics. Because of the indication that NaCl at 0.8 M was suppressing LktA production without affecting growth, it was decided to examine antibiotics which, like osmolarity, are shown to effect DNA supercoiling. *P. haemolytica* Ph 2 was grown as above and, after 5 h the bacteria were resuspended in BHIB in the presence of different antibiotics (novobiocin [25-200 μg/ml] and coumeromycin A1 [20-100 μg/ml]) which are known to affect DNA supercoiling and gene expression (Jovanovich and Lebowitz, 1987). The production of LktA was not altered by the presence of different concentrations of these antibiotics at concentration which did not

Fig. 16.

Effect of sucrose, DBHIB and pH on production of LktA.

P. haemolytica Ph2 was grown for 5 h in BHIB and then the bacteria were suspended in BHIB containing different concentrations of sucrose, dialysed BHIB (DBHIB) or in BHIB at different pH values. Incubation was continued for a further 1 h and the cell free culture supernate was taken and resolved by SDS-PAGE and then transferred to nitrocellulose and probed with convalescent serum.

Lane 1: BHIB + 0.1 M sucrose

Lane 2: BHIB + 0.3 M sucrose

Lane 3: BHIB + 0.7 M sucrose

Lane 4: BHIB + 1 M sucrose

Lane 5: DBHIB

Lane 6: BHIB (pH 8)

Lane 7: BHIB (pH 6)

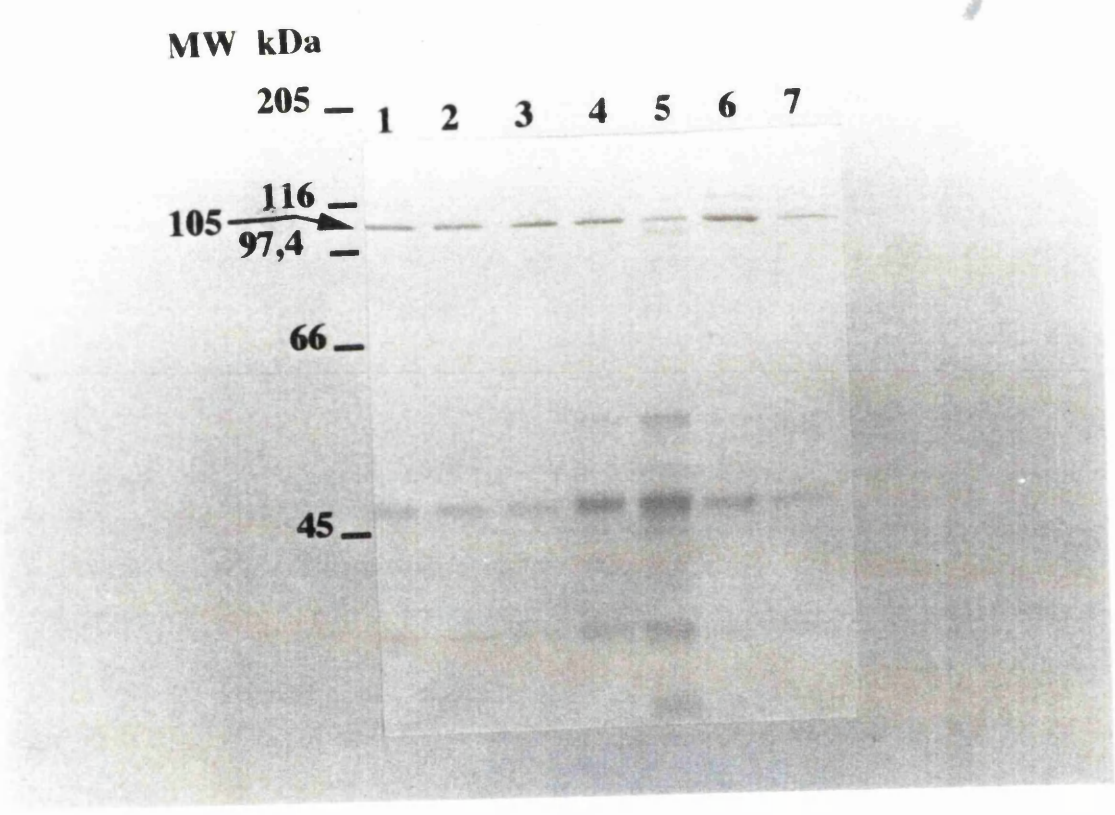

MW kDa
205
1
2
3
4
5
6
7
116
105
97,4
66
45

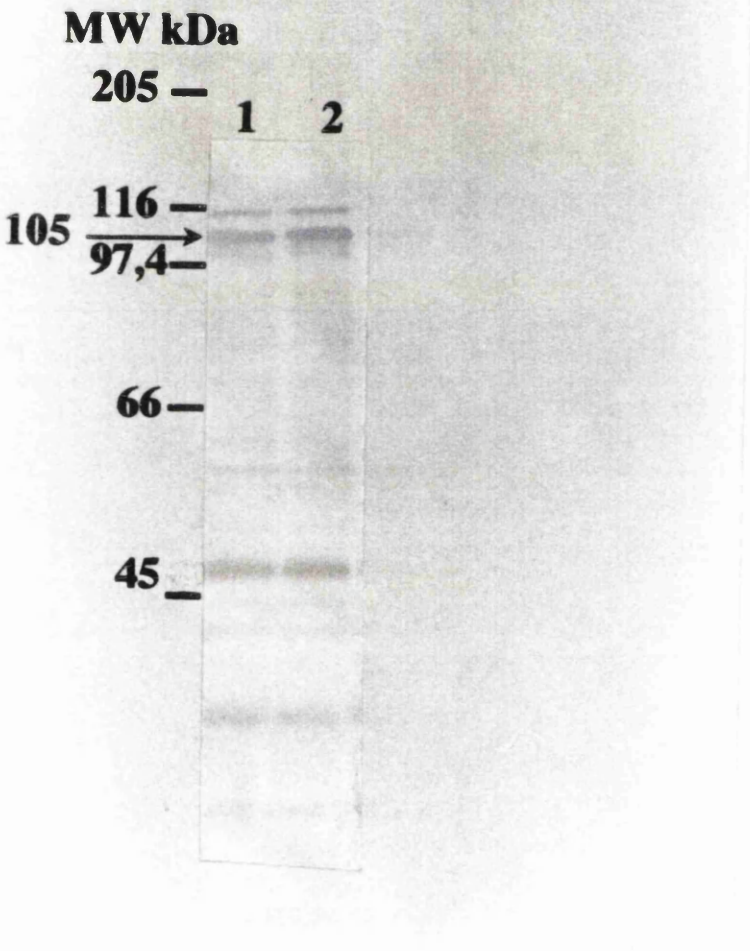
Fig. 17.

The effect of serial passage of *P. haemolytica* on production of LktA.

P. haemolytica Ph 2 was passaged 40 times on BHIA and then was grown in 50 ml BHIB in a 250 ml shake flask at 120 rpm at 37 °C and samples were removed at intervals. The cell-free culture supernates were resolved by SDS-PAGE in a 10% polyacrylamide gel, immunoblotted and probed with convalescent serum raised against Ph2, at dilution of 1 in 200. Arrow shows bands identified as LktA.

Lane 1: culture supernate harvested at 6 h, after 40 subculture

Lane 2: culture supernate harvested at 6 h, after one subculture of cells taken from a glycerol stock


MW kDa
205
1
2
116
105
97,4
66
45

inhibit growth i.e. < 25 μg/ml for novobiocin and < 20 μg/ml for coumeromycin A1 (data not shown).

RPMI medium. In many previous studies on LktA, *P. haemolytica* was grown initially in BHIB, then harvested and resuspended in RPMI with various supplements in order to enhance toxin production (see section 1.5.5). In the present investigation, Ph2 was grown in BHIB for 4 h and then resuspended in RPMI alone or containing various concentrations of FCS (3-14%) or BSA (1-4 mg/ml). After further incubation for 1-2 h, samples were assayed for toxin production and activities. The yield of LktA was greatest in RPMI + FCS 7% after 2 h but this was not markedly greater than the yield obtained by growth in BHIB (data not shown). In view of the technical complexity and expense of the RPMI method, BHIB was chosen for further studies. In addition, the presence of serum protein in RPMI + FCS caused problems in interpretation of gel profiles, caused cross-reactions in immunoblots and also affected CL responses of some species of neutrophils, which did not occur with BHIB as the growth medium.

Dialysed BHIB. The production of toxin in BHIB and Dialysed BHIB (DBHIB) (Chang *et al.*, 1987b) was investigated. *P. haemolytica* was grown in BHIB or DBHIB for 6 h at 37 °C. More toxin was produced in the BHIB than DBHIB (Fig. 16 lane 5).

3.2.1.5 The effect of serial passage of *P. haemolytica* on production of LktA.

P. haemolytica Ph2 was passaged 40 times on BHIA, a process which took 3 months. The production of LktA in culture supernates was measured by the CL-inhibition assay but there was no loss of toxicity by the passaged strain (data not shown). Similarly, serial passage did not affect the quantity of

leukotoxin (LktA) protein present in these samples as judged by immunoblotting (fig. 17, lanes 1 and 2). These results indicate that freshly isolated cultures of this organism are not necessary for toxin production and the bacteria do not lose the ability to produce active LktA.

Thus, in comparing LktA production by different strains of *P. haemolytica*, bacteria were grown in BHIB at 37 °C, with very high aeration and culture supernate samples were taken at the end of log phase. The band at 105 kDa in the crude extract of culture supernate of BHIB and RPMI in SDS-PAGE or blots was identified as the 105 kDa leukotoxin protein. This band also reacted in blots probed with anti-105 kDa *P. haemolytica* leukotoxin monoclonal antibody. In some samples, bands of mol. wt lower than 105 kDa were present indicating possible degradation of LktA.

3.2.2 Ultrafiltration

In an attempt to purify the LktA from BHIB culture supernates, different Amicon ultrafiltration membranes (mol. wt cut-offs 50,000, 100,000 and 300,000) were used. Although the mol. wt of leukotoxin is 105 kDa, as judged by SDS-PAGE, it did not pass through an Amicon ultrafiltration membrane with a cut-off of 300 kDa. The LktA appears therefore to be combined with some other cell component or in an aggregated state. To try to remove the aggregation, the effect of various agents was tested (fig 18). Thus, LktA was treated with 2% SDS (lane 4), 2% zwittergent (lane 6), 1 mM EGTA (lane 8) and 8 M urea. They had no effect on the aggregation of LktA as judged by the failure of the treated toxin to pass through a 300,000 cut-off Amicon ultrafiltration membrane and when the membranes were washed with distilled water, the LktA was detected in the washing fluid which confirmed that it had not passed through the membrane (lanes 2, 3, 5, 7 and 9 of fig. 18)

Fig. 18.

Effect of various agents on LktA aggregation.

P. haemolytica was grown for 6 h at 37 °C in BHIB. Cell-free culture supernate was concentrated 10-fold with an Amicon ultrafiltration membrane (mol wt. cut-off 300,000) at 4 °C. Various agents were tested for their effects on LktA aggregation. LktA was treated with 2% SDS, 2% zwittergent, or 1 mM EGTA but none allowed the LktA to pass through the ultrafiltration membrane. When the LktA was concentrated with an mol. wt 300 Amicon ultrafiltration membrane, the membrane washed with zwittergent and distilled water and LktA was detected with immunoblotting. Ten µl of a 10-fold concentration of culture supernate and washed membrane were resolved on 10% SDS-PAGE and then transferred to nitrocellulose and probed with convalescent serum at a dilution of 1 in 400.
In another experiment, concentrated LktA was passed through the detoxigel. The culture supernate which either bound to detoxigel or passed through the detoxigel was resolved on 10% SDS-PAGE, immunoblotted and probed with convalescent serum at a dilution of 1 in 400.

Lane 1: 10-fold concentration of LktA

Lane 2: membrane washed with distilled. water

Lane 3: membrane washed with zwittergent

Lane 4: 10-fold concentration of LktA + 2% SDS

Lane 5: membrane washed with 2% zwittergent

Lane 6: 10-fold concentration of LktA + 2% zwittergent

Lane 7: membrane washed with distilled water

Lane 8: 10-fold concentration of LktA + 1 mM EGTA

Lane 9: membrane washed with distilled water

Lane 10: the culture supernate which bound to detoxigel

Lane 11: the culture supernate which passed through the detoxigel

Lane 12: the culture supernate which passed through the ultrafiltration membrane

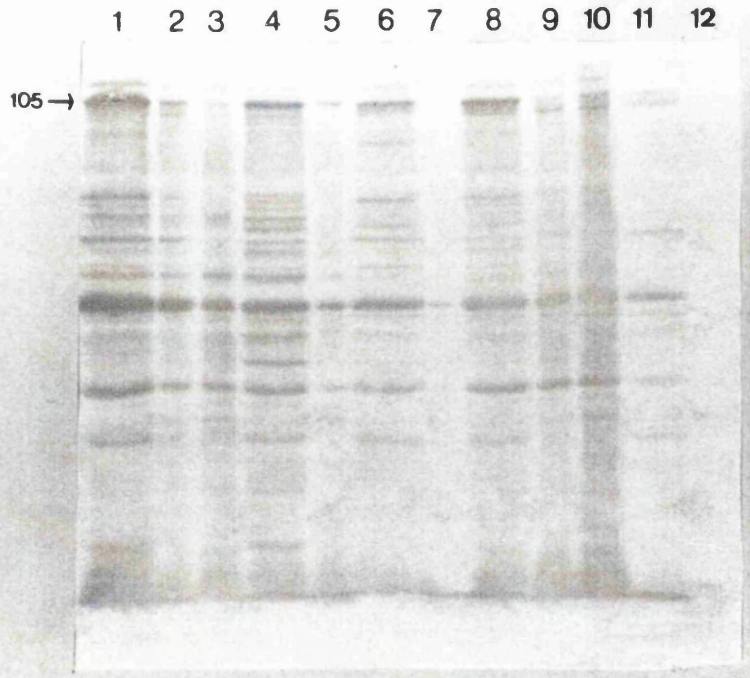

1 2 3 4 5 6 7 8 9 10 11 12
105

Fig. 19.

Preparation of recombinant leukotoxin (rLktA)

Active and inactive form of recombinant leukotoxin were produced by transfer of the *lktA* and *lktC* genes to *E. coli*. *E. coli* DH5αF'IQ (pGW42) produced active rLktA of 105 kDa and an additional protein of >120 kDa (lane 1). *E. coli* HMS174 or SY327λ pir (pGW42) produced active LktA with a mol. wt 105 kDa (lane 2).

E. coli SY327λ pir (pGW64) produced inactive rLktA (lane 4). When plasmid pGW78 was transferred to *E. coli* strain SY327λ pir (pGW64), active rLktA was produced and the mol. wt of rLktA did not change (lane 3). 10 μl of urea extract/well was resolved on a 10% of SDS-PAGE gel, immunoblotted and probed with monoclonal antibody raised against rLktA. Culture supernate of Ph2 was used as control (lane 5).

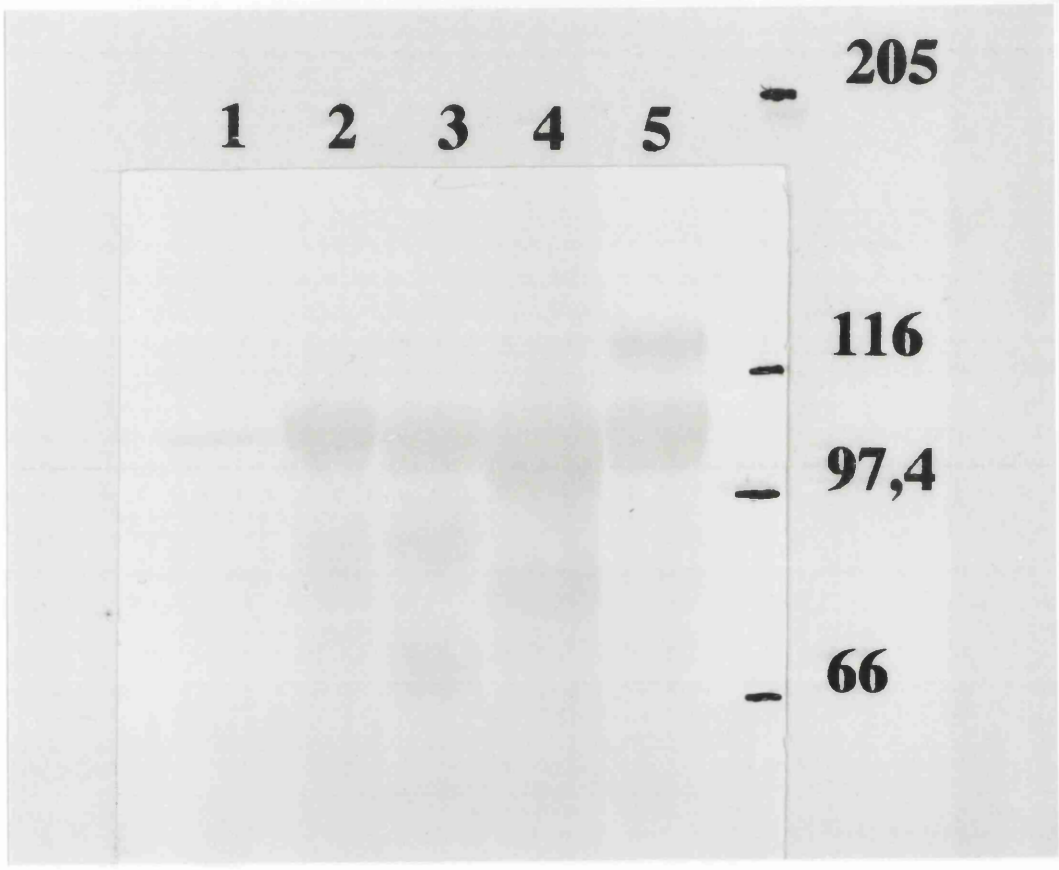
Lane 1: *P. haemolytica* Ph2 culture supernate

Lane 2: *E. coli* strain SY327λ pir + plasmid pGW64 (inactive rLktA)

Lane 3: *E. coli* strain SY327λ pir + plasmids pGW64 + pGW78 (active rLktA)

Lane 4: *E. coli* strain HMS174 + plasmid pGW42 (active rLktA)

Lane 5: *E. coli* strain DH5αF'IQ + plasmid pGW42 (active rLktA)


1
2
3
4
5
205
116
97,4
66

3.2.3 Preparation of recombinant leukotoxin (rLktA)

In order to partially purify and produce large amounts of leukotoxin, the *lktA* and *lktC* genes were cloned into *E. coli*. Different strains of *E. coli* (i.e. *E. coli* strains DH5αF'IQ and HMS174 containing plasmid pGW42, encoding both *lktA* and *lktC* and producing active rLktA, or strain SY327λ pir containing plasmid pGW64 (encoding *lktA*) and/or pGW78 (encoding *lktC*) were used. In DH5αF'IQ, a >120 kDa protein was produced in addition to the 105 kDa by pGW42 (fig. 19, lane 5). This higher molecular weight protein was recognised by a LktA specific monoclonal antibody and was most likely a fusion protein because DH5αF'IQ contains an amber suppressor mutation that would suppress a translational stop codon between *lktC* and *lktA*. *E. coli* strains HMS174 or SY327λ pir containing plasmid pGW42 (encoding active rLkt) produced 105 kDa protein that reacted with the monoclonal antibody raised against rLktA (fig. 19, lane 4). These strains had no suppression or mutations.

E. coli strain SY327λ pir containing plasmid pGW64 produced only inactive leukotoxin with the same apparent mol. wt as the active form of LktA (fig. 19, lane 3). When *lktC* was transferred to the same *E. coli* containing the *lktA* gene, the mol. wt of the resulting, active rLktA did not change (fig. 19, lane 2). Cell-free culture supernate of *P. haemolytica* isolate Ph2 was used as a control (lane 1).

3.3 DEVELOPMENT OF THE CL ASSAY

3.3.1 Different preparations of target cells.

Bovine whole blood, isolated lymphocytes, monocytes and neutrophils were used to measure the normal CL response prior to setting up a CL-inhibition assay. No significant signal was obtained with whole-blood

stimulated with different stimuli, presumably due to quenching of the light output. Small numbers of contaminating erythrocytes can cause significant light arrest and so steps were taken to remove erythrocytes from leukocyte preparations. Percoll or Histopaque at a density of 1.077 was used to separate the mononuclear cells from erythrocytes and neutrophils and the use of isotonic ammonium chloride and hypotonic shock were used for the lysis of the RBCs. Although each method for removing the RBCs had some effect on target cells, the isotonic ammonium chloride method was used routinely. The hypotonic shock very quickly lysed the RBCs, but it reduced the viability of the leukocytes.

In order to find suitable target leukocytes for LktA action and for assay by CL inhibition, the CL response of bovine lymphocytes, monocytes and neutrophils were compared. Bovine lymphocytes 100 µl (5×10^5 ml^{-1}) were treated with DNDH 10^{-5} M and then stimulated with OZ (0.1 mg/ml). Fig. 20 shows that purified lymphocytes did not produce a CL response when incubated with this stimulus. The bovine monocytes produced approx. one-eighth of the CL produced by bovine neutrophils. It was reported that human monocytes produced approx. one-third of the CL signal generated by neutrophils (Nelson *et al.*, 1976).

Two methods were used to prepare the neutrophils (see section 2.5.1). When these two methods, using Percoll and isotonic ammonium chloride, were compared, it was found that neutrophils prepared by lysis of the RBCs with isotonic ammonium chloride were less contaminated with mononuclear cells. Their responses to various stimuli (i.e. PMA OZ, latex beads, fMLP) were similar and leukotoxin bound to and lysed both preparations (see section 3.13.2).

Fig. 20.

Measurement of the normal CL responses of lymphocytes, monocytes and neutrophils

Bovine lymphocytes, monocytes and neutrophils were separated from RBCs by Percoll density gradient centrifugation. Cells (5×10^4 ml^{-1}) were treated with DNDH 10^{-5} M, incubated for 30 min at 38 °C and then stimulated with OZ (0.1 mg/ml).

1: bovine monocytes

2: bovine lymphocytes

3: bovine neutrophils

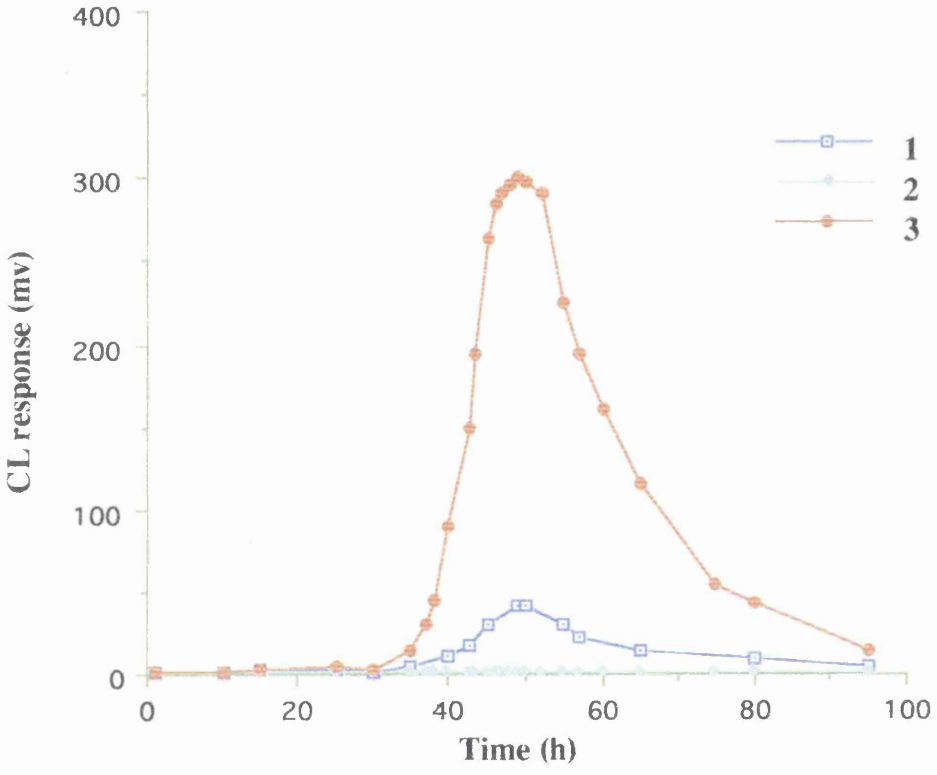

400
300
200
100
0
CL response (mv)
0
20
40
60
80
100
Time (h)
1
2
3

Fig. 21.

Effect of different chemiluminogenic probes on CL response of bovine neutrophils

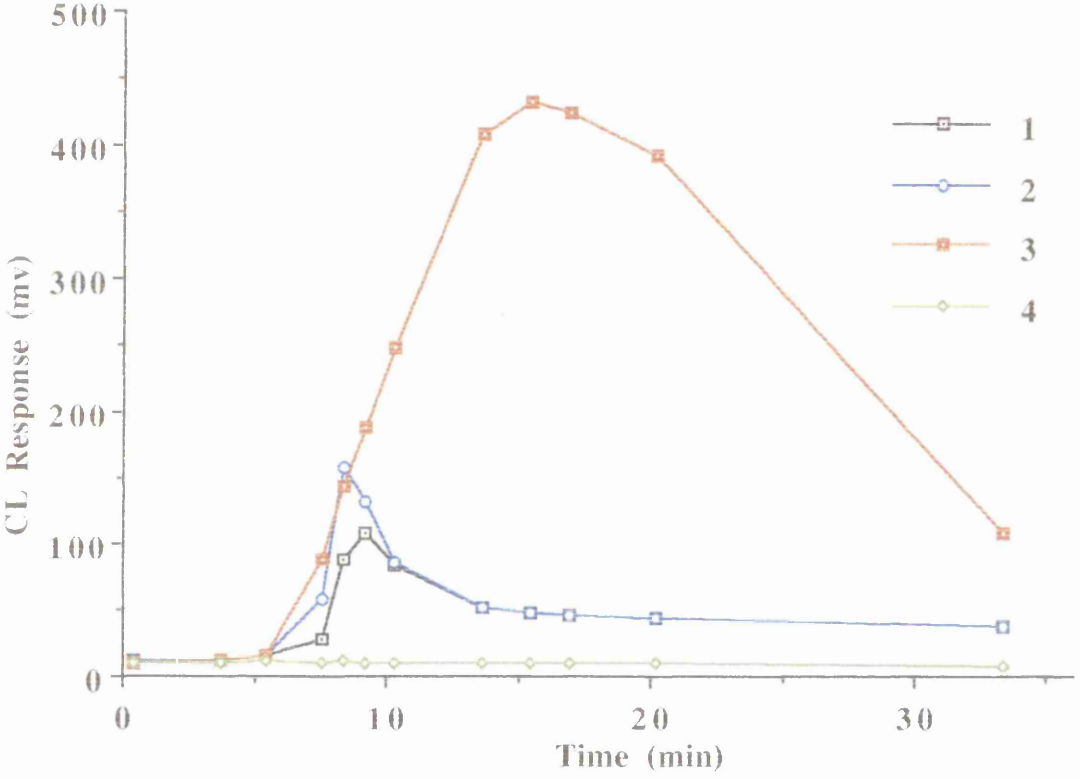
Bovine neutrophils (5×10^4 ml^{-1}) were incubated with 10^{-5} M luminol, lucigenin or DNDH for 5 min at 38 °C. The cells were stimulated with OZ (0.1 mg/ml). The CL response was measured at different times.

1: luminol + PMA

2: DNDH + PMA

3: DNDH + OZ

4: lucigenin + OZ


500
400
300
200
100
0
CL Response (mv)
0
10
20
30
Time (min)
1
2
3
4

3.3.2 Effect of different chemiluminogenic probes on CL response.

The chemiluminescent signal is normally amplified by adding compounds such as luminol, DNDH or lucigenin to the assay mixture. Bovine neutrophils were incubated with different chemiluminogenic probes and then stimulated with OZ or PMA. The results in fig. 21 show that the best chemiluminogenic probe was DNDH, giving a far greater and more prolonged CL response particularly with OZ, than either the luminol or lucigenin (fig. 21).

3.3.3 Effect of different stimuli

Different stimuli can be used to activate different signalling pathways in neutrophils in order to generate a CL response. The chemiluminescence responses of bovine neutrophils to different stimuli i.e. opsonised zymosan, PMA, *f*MLP, latex beads and zymosan were measured. Bovine neutrophils were mixed with DNDH at 38 °C and then stimulated with different stimuli. Fig. 22 shows *f*MLP could not stimulate CL response of bovine neutrophils and latex beads 10^8/ml (particle diameter, 1.16 μ; Sigma) were weak but PMA induced an obvious and rapid response. Opsonised zymosan induced a high, persistent response. The response of the neutrophils to zymosan was lower than to opsonised zymosan. Although cold OZ produced more stimulation of neutrophils, to prohibit the effect of temperature on the CL response, OZ at room temperature was found better for these experiments. Increasing the concentration of stimuli (i.e. OZ, PMA) per assay, caused a greater CL response but OZ at a concentration of 0.1 mg/ml was found to be suitable for subsequent work.

Fig. 22.

Effect of different stimuli on the CL response of bovine neutrophils

Bovine neutrophils (5×10^4 ml^{-1}) were suspended in PBS and then stimulated with different stimuli, PMA (1 μg/ml), fMLP (10^{-7} M), latex beads 10^8/ml, zymosan or OZ (100 μg/ml) in the presence of DNDH 10^{-5} M.

1: opsonised zymosan

2: zymosan

3: fMLP

4: latex beads

5: PMA

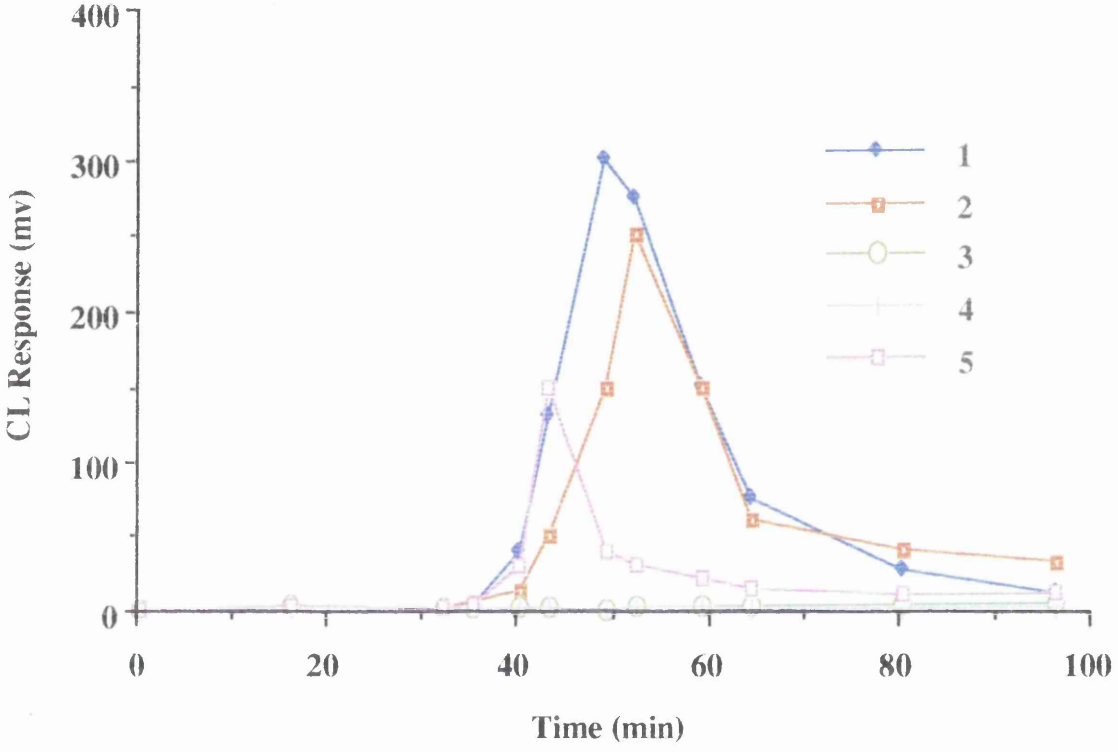

400
300
200
100
0
CL Response (mv)
0
20
40
60
80
100
Time (min)
1
2
3
4
5

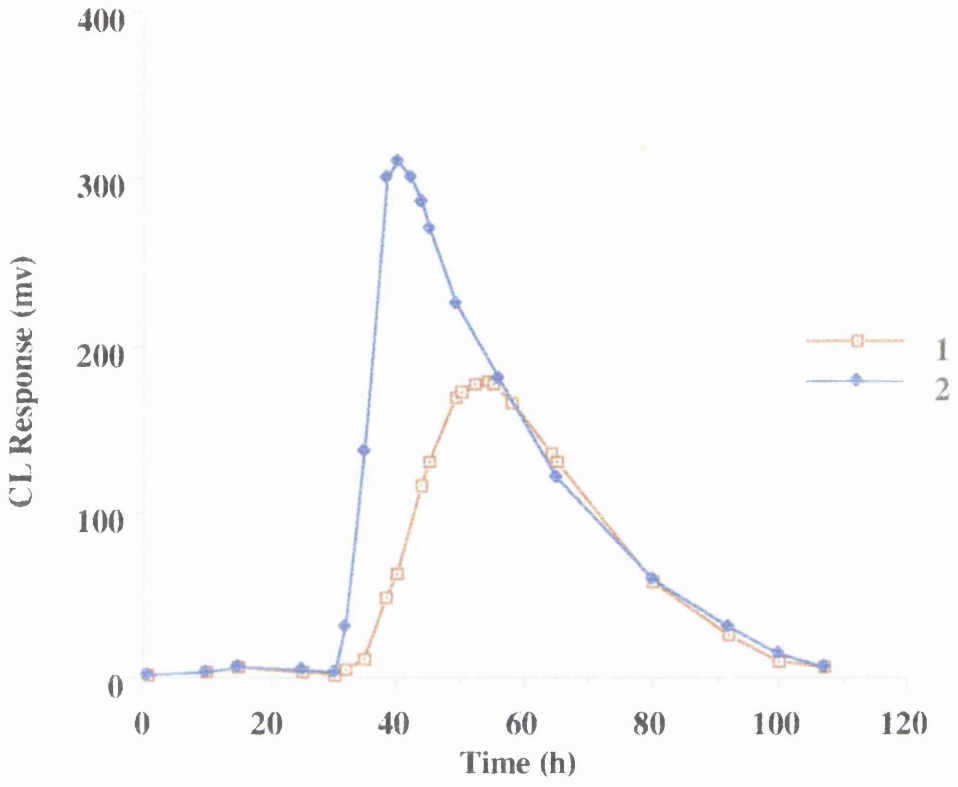
Fig. 23.

Effect of different suspending buffers

Bovine neutrophils were suspended in PBS or HH in the presence of DNDH 10^{-5} for 30 min at 38 °C. The cells were stimulated with OZ (100 µg/ml).

1: PBS

2: HH


400
300
200
100
0
CL Response (mv)
0
20
40
60
80
100
120
Time (h)
1
2

3.3.4 Effect of different suspension buffers

The effect of different suspension buffers (PBS and Hanks Hepes (HH)) on the CL response of neutrophils was examined. Bovine neutrophils were suspended in PBS or HH with DNDH and stimulated with OZ. Fig. 23 shows that the neutrophil response to OZ was greater when suspended in HH. This high response could be due to the presence of 1 mM calcium in this buffer (see appendix. 4).

For assay of LktA, toxin samples and neutrophils were incubated together for different times before the addition of OZ. Incubation for 30 min was found to be most suitable to allow any toxin activity on the target cells, and for the system to equilibrate.

Different concentrations of urea were tested for their effect on the CL response of neutrophils since some toxin samples were prepared by urea extraction. Bovine neutrophils were incubated with different concentrations of urea (0.01, 0.04, 0.08, 0.1, 0.4, 0.8, 1, 4 and 8 M) in the presenxe of DNDH, then stimulated with OZ. <0.08 M urea had no affect on target cells but >0.08 M urea reduced the CL response and presumably it killed the cells. Thus, for assay of LktA urea extracts of bacteria, the concentration of urea was decreased by dialysis or its concentration was reduced to below 0.08 M by dilution (data not shown).

During initial CL experiments, it became apparent that temperature changes during the CL assay i.e. adding cold reagents to the cells incubated at 38 °C in the luminometer or transferring neutrophils suspension from room temperature to the luminometer at 38 °C, caused a small but detectable CL response. To avoid any effects of temperature changes (room and machine) all cell suspensions were equilibrated and kept in the machine during the preincubation time and addition of cold reagents was avoided.

3.4 ASSAY OF LKT IN CULTURE SUPERNATES OF DIFFERENT STRAINS OF *P. HAEMOLYTICA*

The above investigations of factors affecting LktA production by *P. haemolytica* (seesection 3.2.1 and 3.2.2) were done with the serotype A1 bovine isolate, Ph2. Similar results were obtained with strains Ph30, Ph42, FT4, Ph144, Ph146, Ph152 and Ph154, which included serotypes A1, A2, T and untypables. In section 3.2.1.1, it was established that LktA production was maximal at the end of the log phase of growth in BHIB in shaken (120 rpm) cultures at 37 °C. A comparison of the production of LktA by other strains was made using the above growth conditions. Having established suitable conditions for generating a CL response in bovine and ovine neutrophils, different samples could be assayed for LktA activity in a CL inhibition assay. Cell-free culture supernates of *P. haemolytica* isolates, containing LktA, were incubated with bovine and ovine neutrophils for 30 min, in the presence of DNDH, and neutrophils were stimulated by OZ. The activity of the toxin was measured by inhibition of the normal CL response of the neutrophils. The results are summarised in table 4. Culture supernate samples from most strains (21/33) were highly toxic >80% inhibition of the normal CL response of bovine and/or ovine neutrophils (table 4). Samples from an untypable strain, UT3, proved to be the most toxic and were the only samples to give 100% inhibition of CL. Three strains were moderately toxic (41-80% inhibition) for bovine and/or ovine neutrophils. Eight strains had low toxicity (<40% inhibition) for both bovine and ovine neutrophils: these included one serotype A1, two A2, all biotype T (*P. trehalosi*) and one untypable strain. Only one strain, Ph6, a bovine isolate of serotype A1, produced no detectable toxic activity. With all strains, culture supernate samples taken earlier or later during growth did not show any greater toxic activity. Results of a typical CL-inhibition assay, with samples from three

Table 4.
Summary of Lkt activity of *P. haemolytica* isolates and *E. coli* examined by CL assay

Laboratory designation	Serotype	Species of origin	Age of Culture (h)•	Leukotoxic activities†		% of Haemolytic activity
				Bovine	Ovine	
*Ph 2	A1	Bovine	6.00	97	97	48
Ph 10	A1	Bovine	6.00	95	96	40
Ph 8	A1	Ovine	6.00	67	89	23
Ph 12	A1	Bovine	8.00	85	90	31
Ph 14	A1	Bovine	8.00	6	20	16
Ph 26	A1	Bovine	8.00	84	90	36
Ph 30	A1	Bovine	5.30	50	75	27
Ph 6	A1	Bovine	6.00	0	0	2
Ph 48	A1	Ovine	7.30	78	85	17
Ph 42	A2	Bovine	8.00	6	26	33
Ph 44	A2	Bovine	8.30	42	66	41
Ph 72	A2	Ovine	8.30	5	22	13
Ph 140	A2	Ovine	8.00	61	85	23
Ph 142	A2	Ovine	8.00	18	85	15
Ph 50	A5	Ovine	6.30	95	97	33
Ph 52	A6	Ovine	6.30	94	95	25
Ph 54	A7	Ovine	6.30	95	95	23
Ph 56	A8	Ovine	7.00	93	94	49
Ph 58	A9	Ovine	6.30	96	97	46
Ph 60	A11	Ovine	5.00	45	60	22
Ph 62	A12	Ovine	4.30	90	92	45
Ph 64	A13	Ovine	6.30	91	94	22
Ph250	A13	Ovine	5.30	90	90	42
Ph 66	A14	Ovine	5.30	91	94	39
Ph 706	A16	Ovine	6.00	92	94	35
Ph 68	T3	Ovine	6.00	15	32	28
FT 4	T4	Ovine	4.30	36	40	11
Ph 252	FT10	Ovine	4.30	20	35	15
Ph 70	T15	Ovine	4.30	25	39	23
Ph 144	UT	Bovine	3.30	75	82	21
Ph 146	UT	Bovine	5.30	100	100	35
Ph 152	UT	Bovine	4.30	30	39	33
Ph 154	UT	Bovine	5.00	84	85	25

1. * *P. haemolytica*
2. *P. multocida* NCTC 10322 — 0% — 0%
3. *E. coli*
   a. rLktA inactive toxin — 0% — 0%
   b. rLktC — 0% — 0%
   c. rLktA + rLktC active toxin (the same plasmid) — 100% ‡ — 100%
   d. rLktA + rLktC active toxin (different plasmids) — 98% — 98%

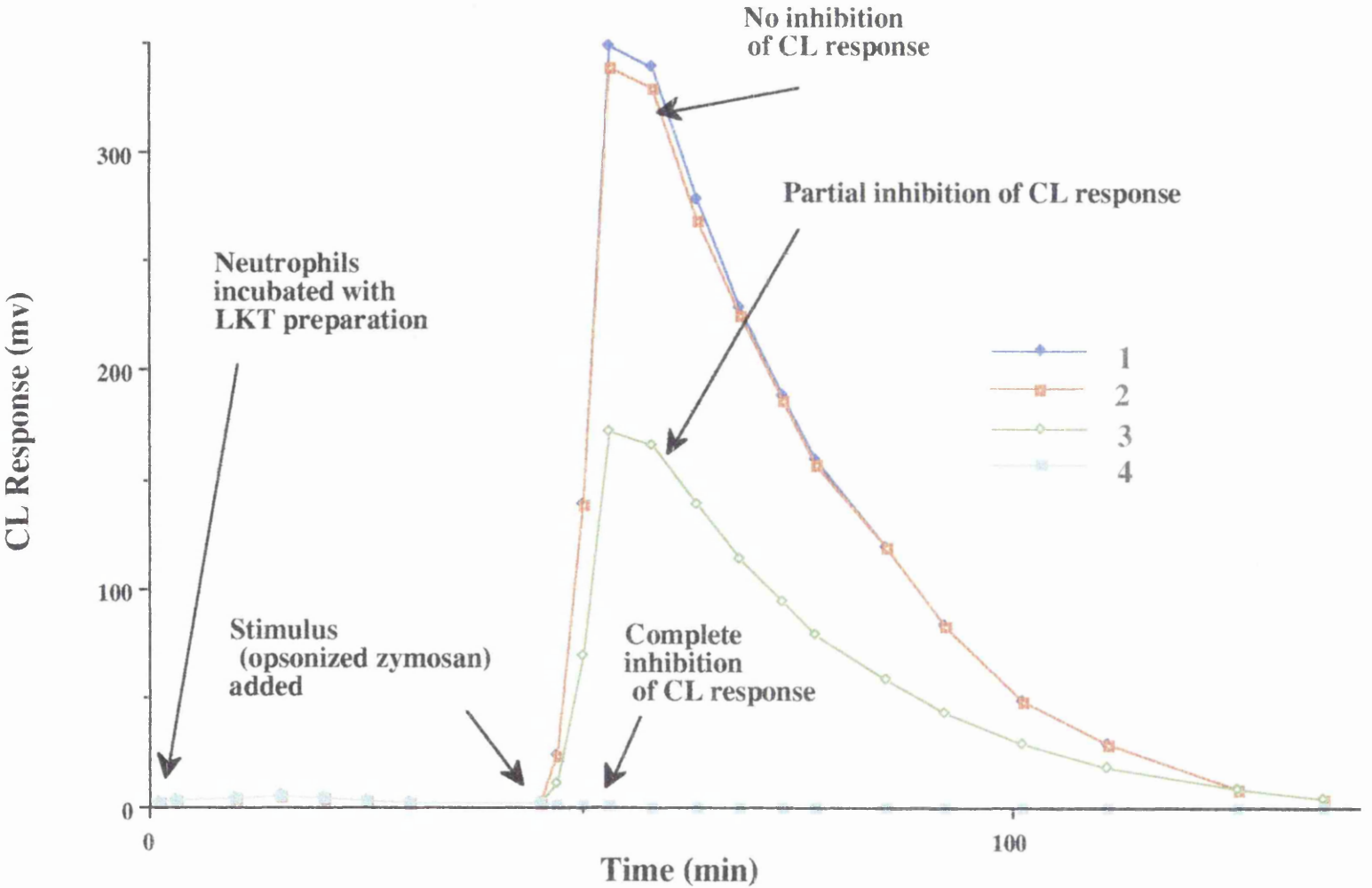
† % inhibition of CL response of neutrophils
• Time of end of exponential phase of growth
‡ At 1/5000 dilution

Fig. 24.

Influence of *P. haemolytica* LktA preparations on CL response of bovine neutrophils

Results of a typical CL-inhibition assay, with samples from three strains of *P. haemolytica* are shown. Bovine neutrophils were incubated for 40 min at 38 °C with late log-phase culture supernate samples from Ph2, Ph30 or Ph72. The cells were then stimulated with 100 µg OZ. Neutrophils in BHIB alone served as a control.

1: BHIB alone (no LktA sample)
2: culture supernate of Ph72
3: culture supernate of Ph30
4: culture supernate of Ph2


No inhibition
of CL response
Partial inhibition of CL response
Neutrophils
incubated with
LKT preparation
Stimulus
(opsonized zymosan)
added
Complete
inhibition
of CL response
1
2
3
4
CL Response (mv)
300
200
100
0
0
100
Time (min)

strains of *P. haemolytica*, are shown in fig. 24. The culture supernate of strain Ph2 was highly toxic and caused almost complete inhibition of CL, that from strain Ph30 caused about 50% inhibition whereas that from strain Ph72 had little effect.

When ovine and bovine neutrophils were compared in terms of their sensitivity to the different toxin preparations, as shown by inhibition of the normal CL response (table 4) there was no significant difference ($p=0.22$, Student's t test). Similarly, a comparison of the ovine and bovine isolates of *P. haemolytica* showed that there was no significant difference ($p=0.29$) in their CL-inhibitory effects on neutrophils (bovine and ovine) .

Culture supernate samples containing LktA showed a reduction in toxic activity on standing at room temperature or at 4 °C and even on prolonged storage. The culture supernate of *P. haemolytica* LktA in BHIB, BHIB + BSA or RPMI + FCS lost toxicity with an increase in temperature.

Cell-free culture supernate from *P. haemolytica* isolate Ph2 grown in BHIB was aliquoted and stored at different temperatures and for different time and then toxicity was measured by the CL-inhibition assay. After 7.5 h at room temperature, the sample lost more than half of its toxicity. The LktA even lost toxicity on prolonged storage at -70 °C (table 5) but the toxicity did not decrease noticeably within the first 10 days. Repeated freeze-thaw treatment decreased the LktA activity. The culture supernate was stored in microcentrifuge tubes in 1 ml volumes and kept at -70 °C, and each sample was frozen and thawed only once. The activity of rLktA stored in 8 M urea for 2 months at -20 °C was found to have decreased slightly (7%). Thus for comparative purposes, all preparations were tested, or frozen, immediately after harvest. In every assay, a culture supernate sample from strain Ph 2 was used as control and internal standard.

Table 5.

Effect of storage temperature on leukotoxin activity

P. haemolytica Ph2 isolate was grown in BHIB for 6 h at 37 °C. Cell -free culture supernate was removed, aliquoted and stored for different times and different temperatures and then the toxicity was measured by the CL inhibition assay.

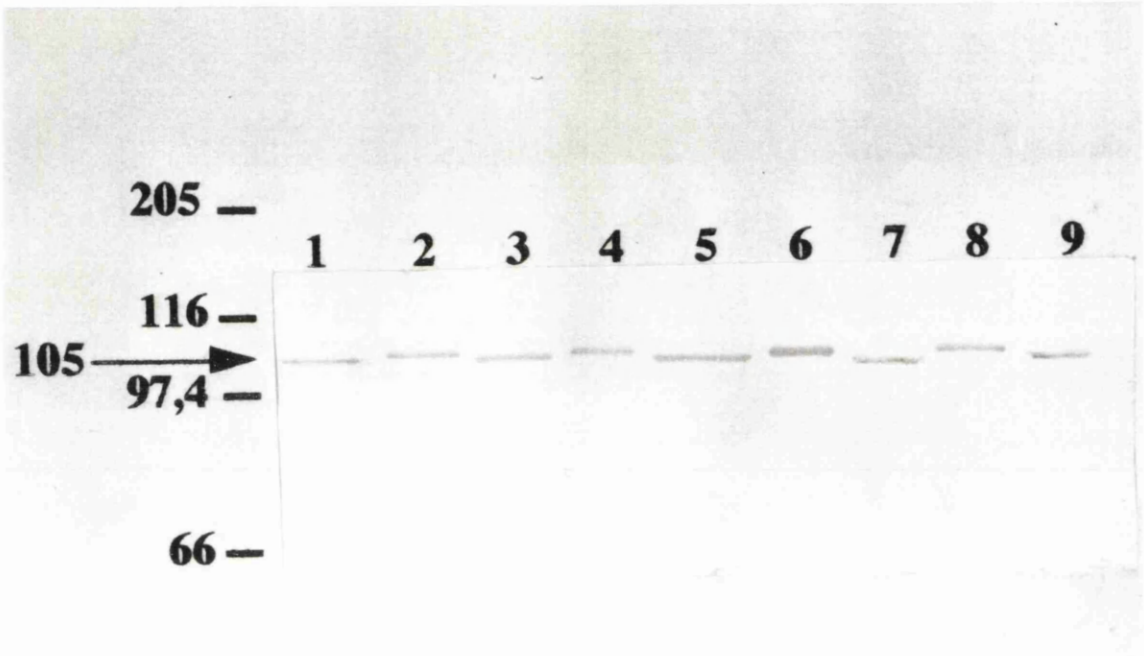
Temperature	Time	% loss of activity
-70 °C	10 days	0
-70 °C	20 days	2
-20 °C	1 day	2
-20 °C	2 days	5
4 °C	1 day	28
4 °C	2 days	38
room temperature	1.5 h	6
room temperature	3.5 h	15
room temperature	4.5 h	32
room temperature	5.5 h	42
room temperature	7.5 h	51
-20 °C (rLkt in urea)	60 days	7

Fig. 25.

Variation in molecular weight (mol. wt) of LktA produced by different strains of *P. haemolytica*

The cell-free culture supernate from the end of log phase of *P. haemolytica* isolates grown in BHIB were resolved by SDS-PAGE (7.5% w/v acrylamide separating gels) and immunoblotted, probed with monoclonal antibody raised against rLktA in a dilution of 1/100. Most isolates (27/33) produced LktA of 105 kDa, as shown by the examples in lanes 1, 3, 5, 7, 9. Four strains (Ph 42, Ph 44, Ph54, Ph152: lanes 2, 4, 6, 8 respectively) produced LktA of 108 kDa

Lane 1: Ph2
Lane 2: Ph42
Lane 3: Ph14
Lane 4: Ph44
Lane 5: Ph48
Lane 6: Ph54
Lane 7: Ph144
Lane 8: Ph152
Lane 9: Ph154


205
116
105
97,4
66
1
2
3
4
5
6
7
8
9

3.5 VARIATION IN AMOUNT AND MOLECULAR WEIGHT (MOL. WT) OF LKT PRODUCED BY DIFFERENT STRAINS OF *P. HAEMOLYTICA*

In addition to screening all isolates for LktA activities, their production of LktA protein was investigated by SDS-PAGE and immunoblotting with monoclonal antibody. All isolates were shown to produce leukotoxin in culture supernates except Ph6 (table 4). The gel and blot revealed that there was some variation in the mol. wt of LktA produced by different strains of *P. haemolytica*. Most isolates (27/33) produced LktA of 105 kDa, as shown by the examples in fig. 25 lanes 1, 3, 5, 7, 9. Four strains (Ph42, Ph44, Ph54, Ph152; lanes 2, 4, 6, 8 respectively) produced LktA of 108 kDa. All of the samples were tested for toxin activities against both bovine and ovine neutrophils in the CL inhibition assay as described in table 4. However, there was no clear relationship between the LktAs with the higher mol. wt and their toxic activities or the characteristics of the producing strains (table 4 and fig. 25). Thus, Ph42 is a bovine A2 isolate from pneumonic lung and its culture supernate had low toxicity; Ph44 is a bovine A2 strain but from the nasopharynx of a healthy animal and the sample had moderate toxicity; Ph54 is an ovine A7 isolate from a pneumonic lung and its culture supernate was highly toxic and a bold LktA band was evident in the blot; Ph152 is an untypable bovine lung isolate and had low toxicity.

Surprisingly, there was no direct relationship between the amount of LktA protein in the culture supernate, as judged by immunoblotting and leukotoxic activities. The culture supernate samples in lanes 1, 5, 6, 7 were highly toxic in the CL assay, that in lane 4 was moderately toxic whereas those in lanes 2, 3, 8 had low toxicity but appeared to contain at least as much LktA protein as the more toxic samples. This was confirmed by ELISA. For example, the sample from isolate Ph42 contained more of the 105 kDa

Table 6. Quantitation of Lkt by ELISA

Lkt preparation from strain:	Amount of Lkt antigen (ELISA units ml^{-1})	Lkt activity (% inhibition of CL response of neutrophils)	
		bovine	ovine
Ph2	100	97	97
Ph42	150	6	26
Ph14	84	6	20
Ph48	74	78	85
Ph54	81	95	95
Ph146	<10	100	100
Ph58	200	96	97
Ph152	12	30	39

LktA protein than strain Ph2 as determined by ELISA (150 and 100 Units of LktA antigen/ml, respectively) but the former had little leukotoxic activity on bovine and ovine neutrophils, whereas the latter was highly toxic (table 6).

3.6 ACTIVITY OF RLKT

The activity of rLktA produced from *E. coli* was measured by CL-inhibition assay. The LktA was extracted with urea from *E. coli*, where it was hyperproduced as inclusion bodies. Urea extract of rLktA was incubated with bovine neutrophils for 45 min and then stimulated with OZ. These urea extracts were typically 5,000-times more active than late log-phase culture supernates of *P. haemolytica*, e.g. strain Ph2. Active rLktA, produced by *E. coli* strains containing plasmid pGW64 + pGW78 or pGW42 alone inhibited the CL response of the neutrophils to OZ at a concentration of at 1 μg protein/ml. However, the inactive rLktA at the same protein concentration or higher (1-35 μg/ml) alone stimulated a CL response when mixed with neutrophils i.e. before the addition of OZ. The subsequent CL response to OZ was not affected i.e. there was no CL inhibition. To show any effect of rLktC on CL response of neutrophils, the rLktC preparation from *E. coli* containing plasmid pGW78 alone was incubated with bovine neutrophils. Fig. 26 shows that rLktC stimulated the neutrophils at time 0 min, i.e. before addition of OZ, and had no effect on the subsequent CL response. In all experiments, urea at the same concentration as in the rLktA preparation, was used as a control. The production of active rLktA protein from *E. coli* strains HMS174 or SY327λ pir with one plasmid containing *lktA* and *lktC* (pGW42) was greater than from *E. coli* strain SY327λ pir with 2 plasmids encoding *lktA* and *lktC* separately (pGW64 + pGW78) (fig. 26).

Fig. 26.

Influence of rLktA preparations on CL response of bovine neutrophils.

Urea extracts of *E. coli* containing the active or inactive forms of rLktA or the activator protein were incubated with bovine neutrophils for 45 min at 38 °C in the presence of DNDH and then stimulated with OZ. Active rLktA protein was produced from *E. coli* with one plasmid expressing both the *lktA* and *lktC* genes (pGW42) and from *E. coli* with separate plasmids encoding *lktA* and *lktC* (pGW64 + pGW78). Neutrophils were incubated in presence of dialysed 8 M urea as a control since LktA and LktC was extracted from the producing cells with 8 M urea and dialysed before use. The arrow shows the time of addition of OZ.

1: dialysed 8 M urea

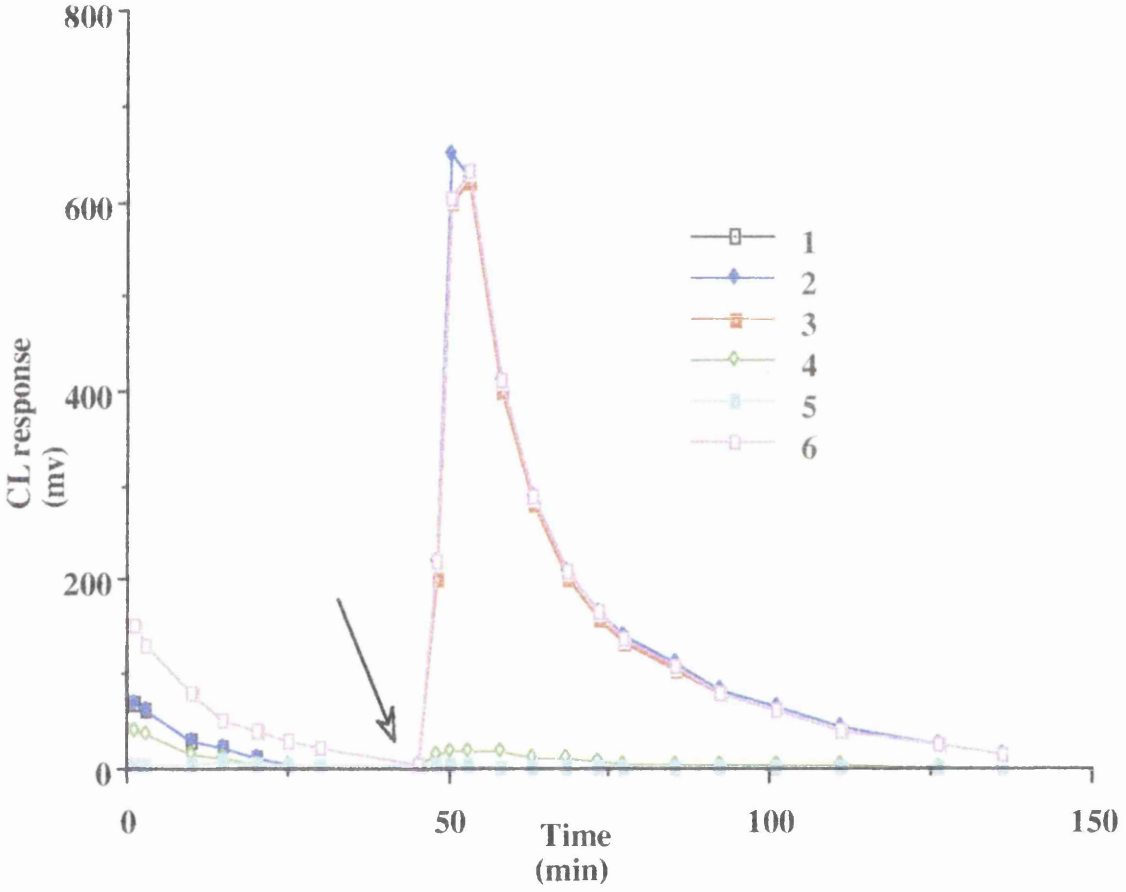
2: dialysed 8 M urea + OZ

3: 1 µg/ml of inactive rLktA (pGW42) + OZ

4: 1 µg/ml of active rLktA (pGW64 + pGW78) + OZ

5: 1 µg/ml of active rLktA (pGW42) + OZ

6: 1 µg/ml of LktC preparation (pGW78) + OZ


800
600
400
200
0
CL response
(mv)
0
50
100
150
Time
(min)
1
2
3
4
5
6

3.7 INFLUENCE OF CALCIUM ON LKT ACTIVITY

In section 3.2.1.4, it was found that growth of *P. haemolytica* strain Ph2 in BHIB in presence of EGTA, in an attempt to deplete calcium ions, had no effect on LktA production, at sub-inhibitory concentrations of EGTA. In other experiments, *P. haemolytica* strain Ph2 was grown in BHIB and different concentrations of EGTA were added to culture supernates after harvesting, to remove any calcium present. This leukotoxin preparation, even with EGTA up to 1 mM was still toxic for bovine neutrophils in the CL-inhibition assay (data not shown) and still bound to erythrocytes in the binding assay (see section 3.1.3.1). The role of calcium ions on the production and cytotoxic activity of native LktA and rLktA was also investigated by growing *P. haemolytica* and *E. coli* containing plasmid pGW42 in Luria-Bertani (LB) broth in the presence of 100 μM EGTA. The bacteria produced leukotoxin in the absence of calcium. The leukotoxin either in the culture supernate or in the urea extracts respectively, was sampled and incubated with bovine neutrophils for 30 min at 38 °C and then stimulated by OZ. No calcium was added at any stage. In neither case was the toxin active in the CL-inhibition assay and the neutrophils were not killed. However, when the neutrophils were incubated with either native or recombinant leukotoxin in the presence of 1 mM calcium, the toxin became active against bovine neutrophils (fig. 27).

3.8 EFFECT OF PROTEASE INHIBITORS AND STORAGE TEMPERATURE

LktA was shown to lose activity fairly rapidly on standing at different temperatures and the possibility that this was due to protease(s) in the LktA preparation was investigated. Thus, the stability of native and recombinant leukotoxin was examined in the presence of a cocktail of protease inhibitors

Fig. 27.

Calcium and cytotoxicity of leukotoxin

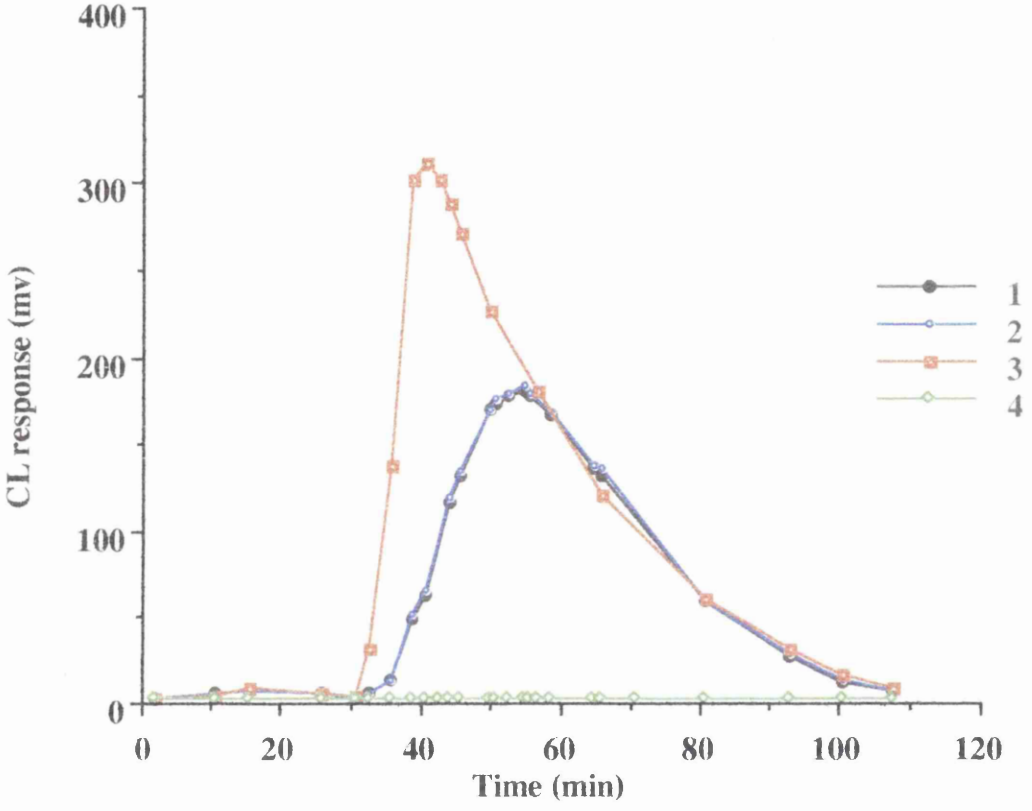
Recombinant LktA was prepared from *E. coli* grown in Luria-Bertani (LB) Broth in the presence of 100 μM EGTA and dialysed urea extracts were incubated with bovine neutrophils for 30 min at 38 °C which were then stimulated by OZ. No calcium was added in any part of the procedure. The toxin was not active, but when 1 mM calcium was added, the toxicity was restored.

1: LB medium (control, without calcium)

2: rLktA without calcium

3: LB medium (control, with 1 mM calcium)

4: rLktA with 1 mM calcium


400
300
200
100
0
CL response (mv)
0
20
40
60
80
100
120
Time (min)
1
2
3
4

(see section 3.13.5) and after storage at room temperature for 24 h. The toxicity of *P. haemolytica* strain Ph2 culture supernate (native leukotoxin) or dialysed urea extract of *E. coli* (rLktA) was measured by CL inhibition assay. The native leukotoxin or rLktA, with or without the cocktail was incubated with bovine neutrophils and then stimulated with OZ. Toxicity of both preparations decreased markedly on standing at room temperature and the cocktail did not preserve toxic activity of either sample.

3.9 SPECIFICITY OF THE CL ASSAY FOR LKT

The specificity of the CL assay for LktA was tested in various ways:

a) *P. multocida* NCTC 10322 was grown on BHIB for 6 h at 37 °C and the culture supernate was removed and incubated with bovine neutrophils for the CL-inhibition assay. This culture supernate, presumably containing cell products common to all *Pasteurella* species, had no effect on the CL response (table 4).

b) To exclude the possibility that the toxicity of culture supernates for neutrophils was due to, or affected by, any contaminating LPS, cell-free culture supernate from *P. haemolytica* isolate Ph2 or a rLktA urea extract of *E. coli* strain HMS174 containing plasmid pGW42 was boiled for 30 min and then incubated with bovine neutrophils for 30 min and then stimulated with OZ. CL inhibitory activity was destroyed (data not shown). LPS is known to be heat stable and would be expected to withstand this treatment.

c) *P. haemolytica* was grown on BHIB at 37 °C until the end of log phase and culture supernate was removed by centrifugation. Release of LPS into the growth medium was shown by resolving the cell-free culture supernate of different isolates of *P. haemolytica* by SDS-PAGE, and immunoblotting with either convalescent serum (fig 14b) or rabbit polyclonal antibody raised against purified LPS from *P. haemolytica* Ph2 isolate (fig.

Fig. 28.

Production of LPS in culture supernates of *P. haemolytica* strains.

Different isolates of *P. haemolytica* were grown on BHIB until the end of log phase i.e. approx. for 6 h at 37 °C. Cell-free culture supernate (50 µl/well) was resolved on 12.5% SDS-PAGE, transferred to nitrocellulose and probed with rabbit polyclonal antibody raised against purified LPS of *P. haemolytica* Ph2 isolate. Purified LPS of *P. haemolytica* Ph2 (1 µg) was used as a control.

Lane 1: LPS control
Lane 2: culture supernate of Ph10
Lane 3: culture supernate of Ph44
Lane 4: culture supernate of Ph706
Lane 5: culture supernate of Ph72
Lane 6: culture supernate of Ph12
Lane 7: culture supernate of Ph58
Lane 8: culture supernate of Ph28
Lane 9: culture supernate of Ph54
Lane 10: culture supernate of Ph30
Lane 11: culture supernate of Ph42
Lane 12: culture supernate of Ph2
Lane 13: LPS control

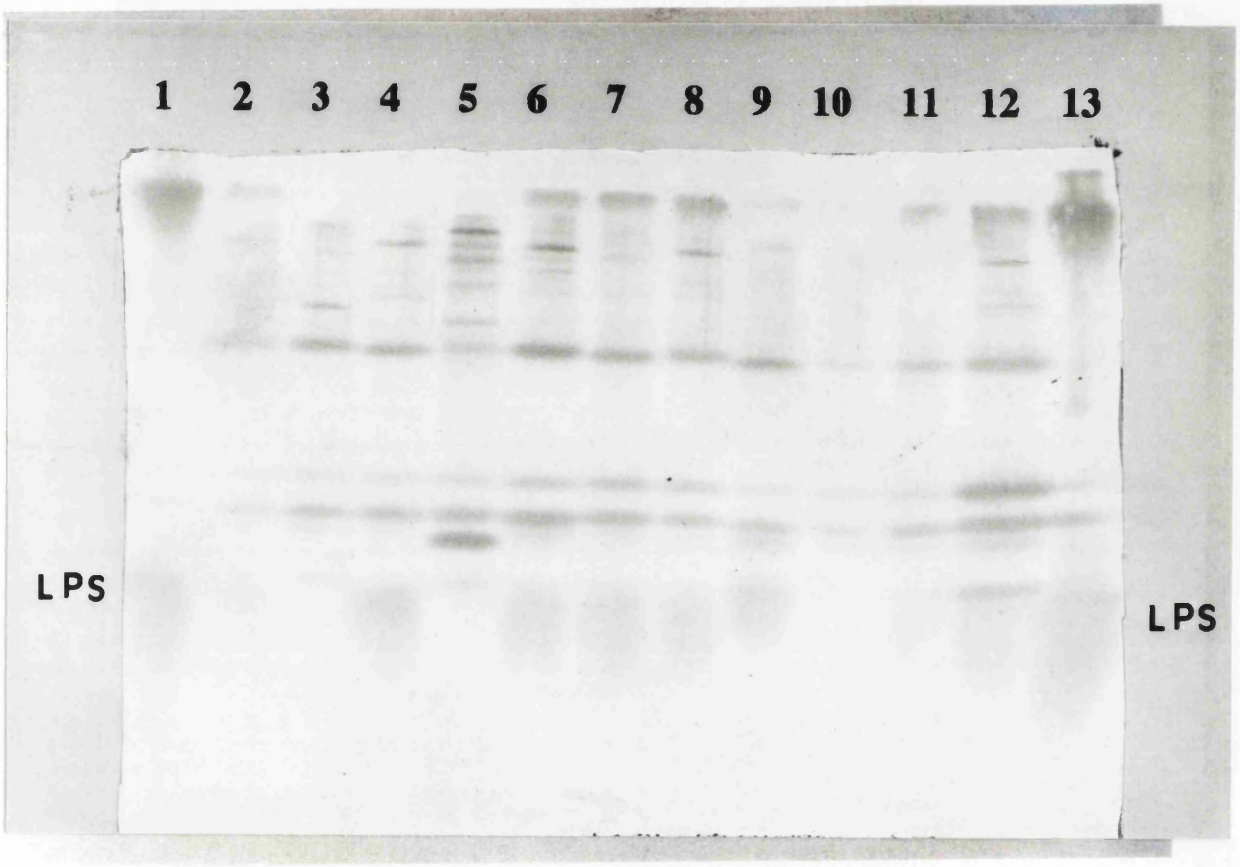

1 2 3 4 5 6 7 8 9 10 11 12 13
LPS
LPS

Fig. 29.

Effect of purified LPS of *P. haemolytica* (rough and smooth) and LPS of *E. coli* (smooth) on bovine neutrophils.

Freeze-dried LPS was suspended in HH and bovine neutrophils were incubated with 400 µg/ml LPS of *P. haemolytica* (rough and smooth) or LPS of *E. coli* (smooth) for 30 min in presence of DNDH 10^{-5} and then neutrophils were stimulated with OZ (0.1 mg/ml). Neutrophils were incubated with HH as a control. The arrow shows the time of addition of OZ.

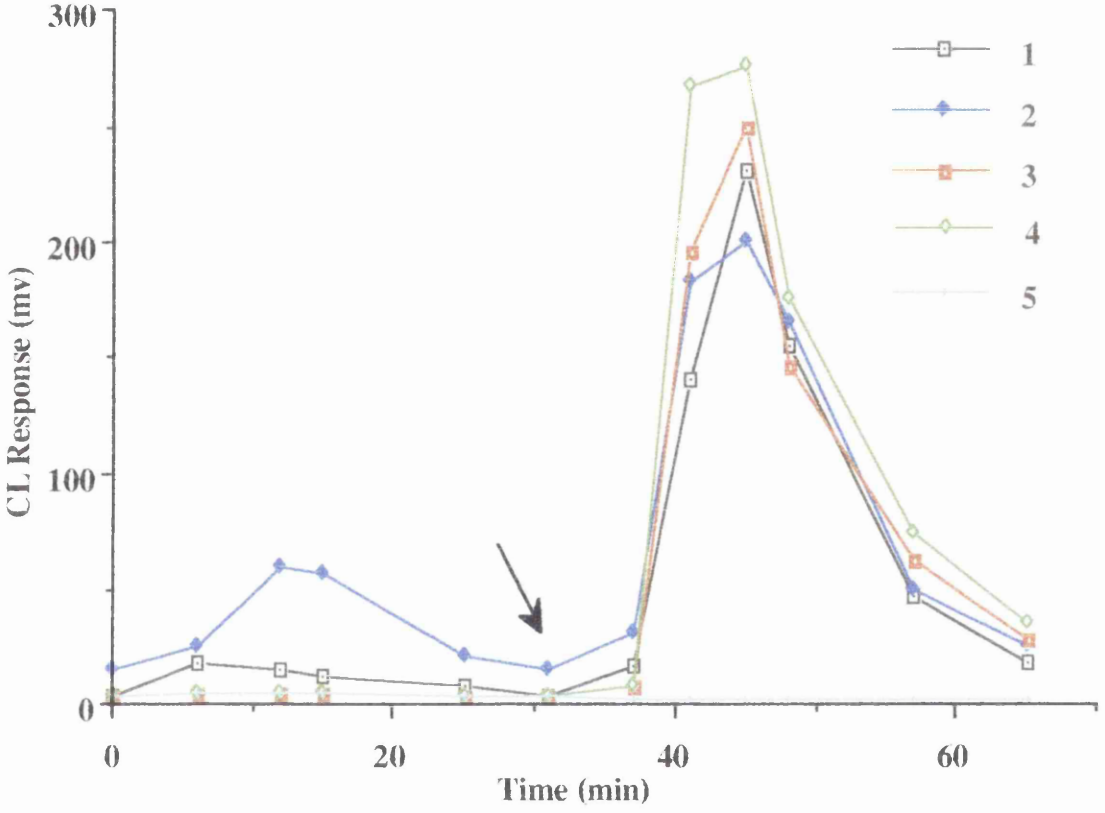
1: *P. haemolytica* smooth LPS + OZ

2: *P. haemolytica* rough LPS + OZ

3: *E. coli* LPS + OZ

4: HH + OZ

5: HH (control)


300
200
100
0
CL Response (mv)
0
20
40
60
Time (min)
1
2
3
4
5

28). Purified LPS type 1 from Ph2 isolate (kindly provided by Dr R Davies) was used as a control (fig. 28 lanes 1 and 13) and high and low mol. wt bands were detected. Corresponding low mol. wt bands were seen in the culture supernate samples of most isolates. The antibody raised against LPS from Ph2 did not cross react with LPS from some strains e.g. strain Ph30 (lane 10). A similar result has been reported (Ali *et al.*, 1992; Ali, 1993). The other bands seen in the immunoblot are presumably due to reaction of the antiserum with proteins produced by the bacteria. The polyclonal antibody raised against purified LPS reacted with these proteins presumably because the rabbit had been exposed to other cross-reactive antigens before bleeding.

The effect of purified LPS of *P. haemolytica* (rough and smooth) and LPS of *E. coli* (smooth) on the bovine neutrophils was investigated. Freeze dried, purified LPS was suspended in HH and bovine neutrophils were incubated with different concentrations up to 400 μg dry wt/ml for 30 min in presence of DNDH 10^{-5} M and then the neutrophils were stimulated with OZ (0.1 mg/ml). The bovine neutrophils were stimulated to some extent by LPS alone and gave a small CL response between time 0 and the addition of OZ (fig. 29). The stimulation by rough LPS of *P. haemolytica* was greater than by smooth LPS of *P. haemolytica*. The subsequent CL response to OZ of bovine neutrophils was only slightly reduced. *E. coli* LPS (smooth) used as a control and at 400 μg/ml also caused slight inhibition of the subsequent CL response (fig. 29). Thus, any LPS present in LktA preparations, from *P. haemolytica* or *E. coli*, is unlikely to be responsible for CL-inhibition attributed to LktA.

d) Further evidence for the specificity of the CL-inhibition assay for LktA was provided when urea extracts of *E. coli* containing rLktA were tested. Only rLktA produced in the presence of LktC was active on the bovine neutrophils (fig. 26). LktA produced in the absence of LktC was

completely inactive, as were urea extracts containing only LktC and urea extracts of the *E. coli* without recombinant plasmids. All rLktA preparations were tested at the same protein concentration (1 μg/ml) and the active and inactive rLktA preparations contained similar amounts of LktA antigen as judged by immunoblotting and ELISA.

e) The CL-inhibitory activity was shown to be inhibited by antibody specific for LktA. Culture supernates of *P. haemolytica* Ph 2 (undiluted) and rLktA (1 in 5,000 dilution) from *E. coli* were incubated for 30 min with polyclonal antiserum raised against purified rLktA (section 3.11) and their toxicity was then measured in the CL-inhibition assay. The antiserum completely inhibited the effect of both preparations of LktA on the neutrophils (fig. 30).

Detoxi-gel (endotoxin-removing gel) (Pierce, Affinity Park™, USA) was used in an attempt to remove the LPS from *P. haemolytica* culture supernates. The toxicity of culture supernate was decreased, as judged by CL-inhibition assay, after detoxi-gel treatment. When the amount of LktA in the samples, before and after detoxi-gel treatment, was examined by immunoblotting, there was an obvious reduction in intensity of the LktA in the band after detoxi-gel treatment, suggesting that detoxi-gel removed LktA as well as LPS.

3.10 COMPARISON OF CL-INHIBITION ASSAY WITH OTHER METHODS OF MEASURING LKT ACTIVITY AGAINST VARIOUS TARGET CELLS.

In addition to the CL-inhibition assay, the effect of leukotoxin on different target cells was measured by other methods: Trypan blue dye exclusion assay, a haemolytic assay (see section 3.12), a chemotaxis assay (see section 3.15) and a cell tracking assay (see section 3.16). It was found

Fig. 30.

Effect of antibody and LktA on CL response.

Culture supernates of *P. haemolytica* Ph 2 (undiluted) or rLktA (1 in 5000 dilution) from *E. coli* were incubated for 30 min with polyclonal antiserum raised against rLktA and then added to bovine neutrophils. The cells and mixture of toxin and antibody were incubated for 25 min at 37 °C in the present of DNDH. Bovine neutrophils were stimulated with OZ. Neutrophils incubated with leukotoxin or antibody alone as controls.

1: polyclonal antibody

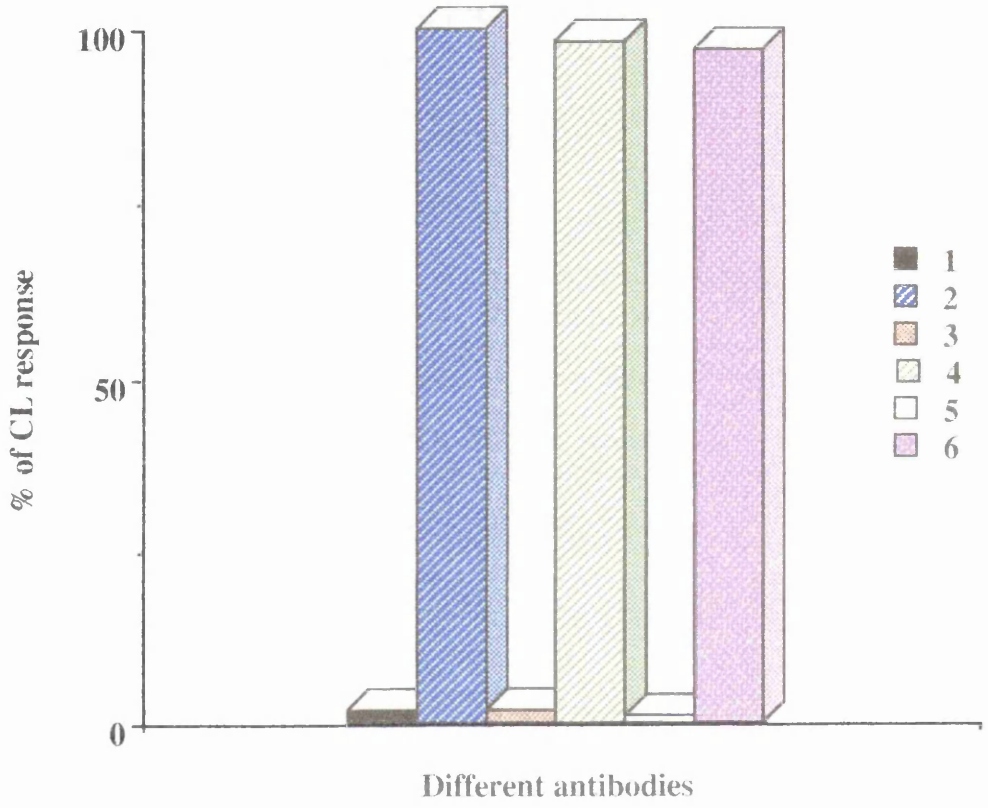
2: polyclonal antibody + OZ

3: *P. haemolytica* Ph2 culture supernate

4: *P. haemolytica* Ph2 culture supernate+ polyclonal antibody

5: rLktA

6: rLktA + polyclonal antibody


100
50
0
% of CL response
Different antibodies
1
2
3
4
5
6

that the Trypan blue dye exclusion assay involved multiple cell counts, was time consuming, prone to error and appeared to be less sensitive than CL-inhibition assay. For these reasons it was not used for comparing LktA in different samples. Trypan blue exclusion was, however, used routinely for checking viability of leukocytes preparations before use.

3.10.1 The effect of LktA on bovine monocytes

Bovine monocytes were incubated with cell-free culture supernate of *P. haemolytica* Ph2 isolate for 30 min in the presence of DNDH and then stimulated by OZ. The activity of the LktA was measured by the CL inhibition assay. Although the response of bovine monocytes to OZ (0.1 mg/ml) in present of DNDH (10^{-5} M) was low, as fig. 20 shows, the LktA preparation was seen to have an effect on bovine monocytes and caused CL-inhibition (see also fig. 45).

3.10.2 Activity of LktA on ruminant and non-ruminant cells in CL-inhibition assay

To investigate the reported specificity of LktA for ruminant cells, different cell types from various ruminant and non-ruminant species were used as targets. The ruminant cells assayed were bovine and ovine neutrophils, which were suitable target cells as reported above and a bovine lymphoma cell line (BL3). These latter cells did not produce a respiratory burst when incubated with DNDH and stimulated with a variety of stimuli i.e. OZ, PMA and latex beads and, so were not used in a CL inhibition assay. However, rLktA killed BL3 cells as judged by Trypan blue (100% killing after 20 min at 37 °C with a concentration of 3 µg protein /ml of rLktA).

The non-ruminant cells assayed were human, rabbit and guinea pig neutrophils, and a mouse macrophage cell line (J774.2).

Fig. 31.

Effect of LktA on CL response of mouse macrophage cell line J774.2

Culture supernate of *P. haemolytica* Ph2 was incubated with mouse macrophage cell line J774.2 in presence of DNDH 10^{-5} M for 35 min. The cells were stimulated with PMA (1 μg/ml). Mouse macrophage cell line J774.2 was incubated with BHIB as a control. The arrow shows the time of addition of PMA.

1: BHIB (control)

2: BHIB + PMA

3: *P. haemolytica* Ph2 culture supernate + PMA

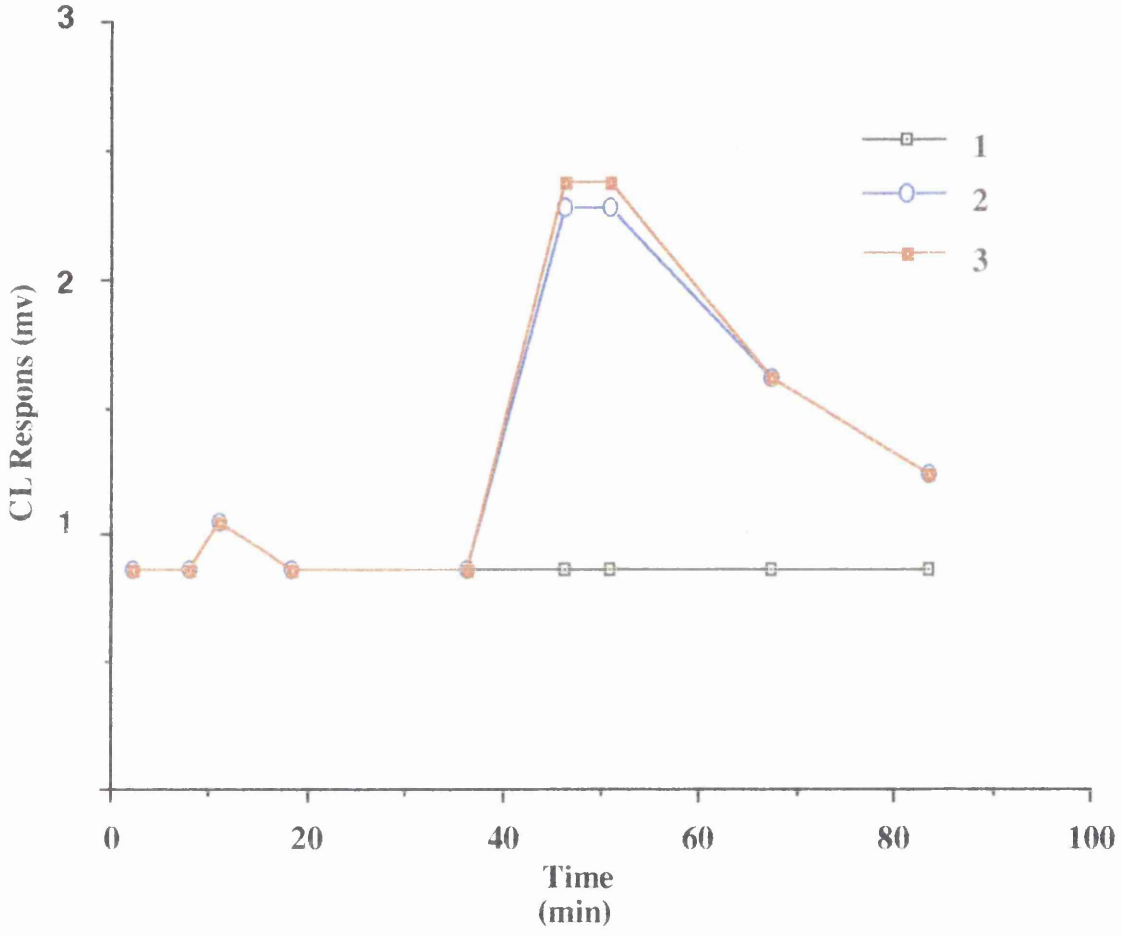

3
2
1
CL Respons (mv)
1
2
3
0
20
40
60
80
100
Time
(min)

Fig. 32.

Effect of native LktA and dialysis of rLktA on inhibition of CL response of human neutrophils.

Late log phase of culture supernate of Ph2 or dialysed urea extracts of rLktA were incubated with human neutrophils (5×10^4) in presence of luminol (10^{-5} M) for 40 min. The cells were stimulate with PMA (1µg/ml). Neutrophils were incubated with BHIB or dialysed 8 M urea as a control.

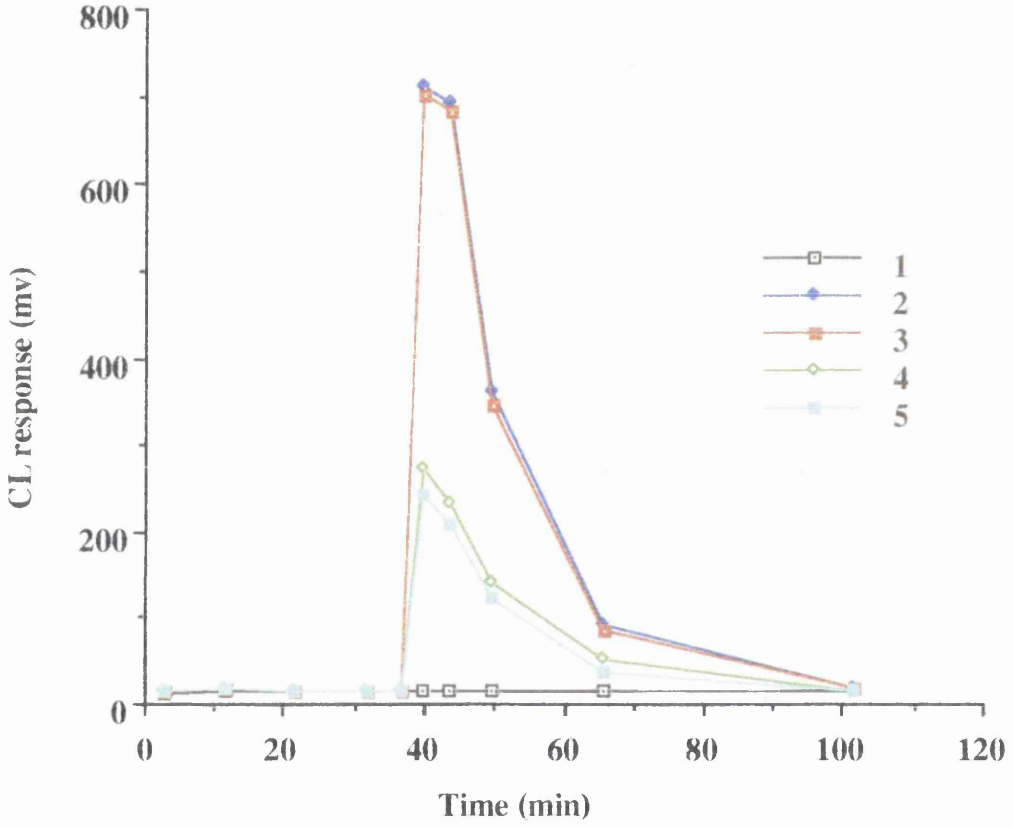
1: urea (control)

2: inactive rLktA produced by *E. coli* SY327λ pir (pGW64) + PMA

3: active rLktA produced by *E. coli* DH5αF'IQ (pGW42) + PMA

4: *P. haemolytica* Ph2 culture supernate + PMA

5: active rLktA produced by *E. coli* SY327λ pir (pGW42)+ PMA


800
600
400
200
0
CL response (mv)
0
20
40
60
80
100
120
Time (min)
1
2
3
4
5

Neither native nor recombinant LktA had any effect on the mouse macrophage cell line (J774.2), which gave a weak but consistent CL response to PMA (fig. 31). Although previous reports have suggested that the leukotoxin is specific for ruminant leukocytes, culture supernates from log phase cultures of *P. haemolytica* Ph2 and Ph10 caused more than 50% inhibition of human neutrophil responses (fig. 32). Recombinant LktA from *E. coli* had different effects. In some preparation of rLktA, the *E. coli* strain used was DH5αF'IQ and in others it was SY327λ pir or HMS174. Active rLktA from *E. coli* HMS174 plasmid (pGW42) or *E. coli* SY327λ pir (pGW64 and pGW78) partially inhibited the CL response. However non-active rLktA (produced in absence of LktC) had no inhibitory or stimulatory effect on human neutrophils. Surprisingly, rLktA from *E. coli* strain DH5αF'IQ, containing plasmid pGW42, did not have any effect on the human neutrophils. This preparation was unusual in other respects i.e. it contained a higher mol. wt component (see fig. 19) which indicated that it was a fusion protein of LktA and LktC. Although this protein was highly active against bovine neutrophils, it was not active against human neutrophils.

The effect of purified LPS of *P. haemolytica* (both R and S) and LPS of *E. coli* (S) on the human neutrophils was investigated. Human neutrophils were incubated with different concentrations up to 400 μg/ml of purified LPS in presence of luminol and then the cells were stimulated with PMA. The human neutrophils were stimulated by LPS alone before the addition of PMA (fig. 33). The stimulation of cells by rough LPS was greater than by smooth LPS of *P. haemolytica*. The subsequent CL response to PMA of human neutrophils was reduced. However, with low concentration of LPS, the LPS had no effect on the CL response. The CL response of neutrophils to culture supernate and LPS before the addition of stimuli were different. The LPS

Fig. 33.

Effect of LPS on the human neutrophils.

Freeze dried preparations of LPS were diluted in HH and human neutrophils were incubated with LPS 1.6 mg/ml of *P. haemolytica* (rough and smooth) and LPS of *E. coli* (smooth) for 30 min in presence of luminol 10^{-5} M. The neutrophils were stimulated with PMA (1 μg/ml). Neutrophils were incubated with HH as a control.

1: HH (control)

2: HH + PMA

3: *E. coli* LPS + PMA

4: *P. haemolytica* smooth LPS + PMA

5: *P. haemolytica* rough LPS + PMA

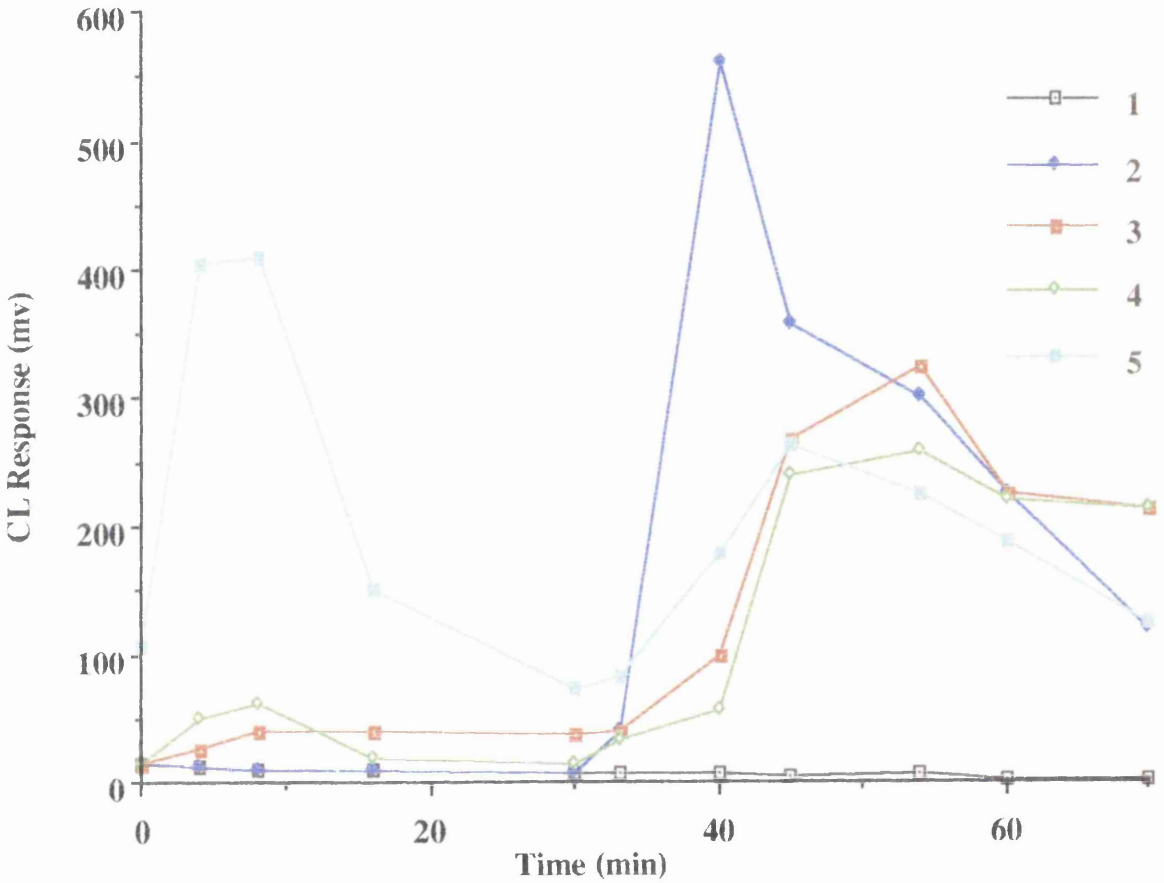

600
500
400
300
200
100
0
CL Response (mv)
0
20
40
60
Time (min)
1
2
3
4
5

Fig. 34.

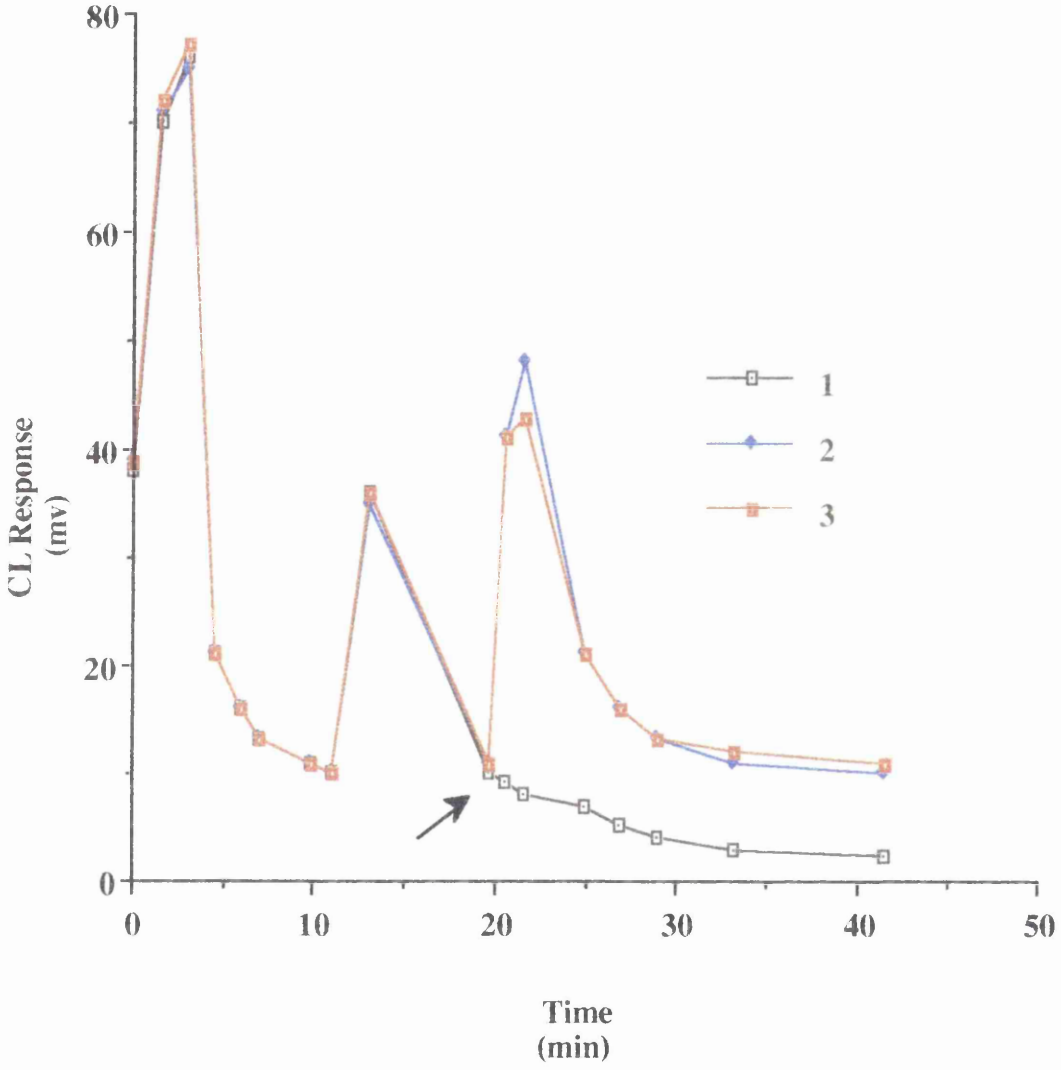
Effect of native LktA on inhibition of CL response of guinea pig neutrophils.

Late log phase culture supernate of Ph2 was incubated with guinea pig neutrophils (5×10^5) in presence of DNDH (10^{-5}) for 40 min. The cells were stimulated with PMA (1µg/ml). Neutrophils were incubated with BHIB as a control. The arrow shows the time of addition of PMA.

1: BHIB (control)

2: BHIB + PMA

3: culture supernate of Ph2 + PMA


80
60
40
20
0
CL Response
(mv)
0
10
20
30
40
50
Time
(min)
1
2
3

stimulated the respiratory burst of human neutrophils (fig. 33) but culture supernate did not (fig. 32).

The LktA preparations had no effect, either inhibition or stimulation, on rabbit neutrophils (table 9). When neutrophils of guinea pig were incubated with DNDH, the neutrophils produced two peaks, the first being bigger than the second. However, the LktA preparations had again no effect on guinea pig neutrophils (fig. 34).

P. haemolytica Ph2 culture supernate was incubated with bovine neutrophils at different temperatures (4 and 37 °C) for 30 min. The cytotoxin of culture supernate of Ph2 was active at 37 °C, but it could not lyse bovine neutrophils at 4 °C as judged by trypan blue dye exclusion.

3.11 TOXIN-NEUTRALISING ANTIBODIES

Neutralisation of the toxic activity of LktA for bovine neutrophils by different antibody preparations was investigated. The BHIB culture supernates (late-log phase) of different strains of *P. haemolytica,* or rLktA from *E. coli* were incubated for 30 min with convalescent serum obtained from cattle experimentally infected with *P. haemolytica* strain Ph2. This serum undiluted, completely neutralised the toxin activities, as judged by the CL-inhibition assay, in all strains tested.

A polyclonal antiserum raised in rabbits against the 105 kDa rLktA (active) purified by preparative SDS-PAGE completely abolished the CL-inhibitory effect of the rLktA and of the culture supernates of *P. haemolytica* A1 (Ph2 and Ph10) on bovine neutrophils. With two *P. haemolytica* strains, however, different results were obtained. This polyclonal antibody partially inhibited the toxicity of culture supernates of *P. haemolytica* NCTC 10634 (Ph54), whereas no inhibition of the sample from Ph146 was seen. Normally, 5 µl sample was mixed with 30 µl neat antiserum to demonstrate neutralisation,

but with the sample from Ph146, no neutralisation occurred even with 90 µl. At high levels of antiserum, the CL response of bovine neutrophils to OZ was affected slightly. The lack of neutralisation of LktA from Ph146 by this antibody was paralleled by the lack of reactivity in ELISA (table 6).

Cell-free culture supernate of Ph2 was incubated with different monoclonal antibodies for 30 min and then incubated with bovine neutrophils. Monoclonal antibodies raised against rLktA, *B. pertussis* AC toxin (9D4), 2-5 (IgG1) raised against AC toxin or *A. pleuroneumoniae* toxin all had slight but different levels of neutralising activity against the toxin at highest doses tested (fig. 35). None of those monoclonal antibodies could completely neutralise the toxin.

3.12 HAEMOLYTIC ACTIVITIES OF DIFFERENT STRAINS.

At the start of this study, it was known that some RTX toxins e.g. *E. coli* haemolysin are strongly haemolytic whereas some e.g. *B. pertussis* AC toxin are weakly haemolytic. The situation with LktA was not clear and some reports suggested that LktA and haemolysin were separately produced by *P. haemolytica* (see section, 1.5.5). The haemolytic activity of *P. haemolytica* isolates was determined in various ways.

First, the haemolytic activity of *P. haemolytica* isolates was determined on sandwich blood agar (BHIA) containing bovine blood at a final concentration of 5% in the top layer. Differences in activities were seen between isolates, e.g. the haemolytic activity of Ph140 isolate was less than that of strain Ph62 (fig. 36a). Similar effects were obtained with human blood (fig. 36b).

The haemolytic activity of *P. haemolytica* on blood plates was weak and attempts were made to improve detection of activity. Selected strains were grown on BHIA plus 5% sheep blood containing different

Fig. 35.

Effect of different antibodies on native LktA and rLktA.

Neutralisation of leukotoxin was measured by incubation of bovine convalescent serum (dilution 1 in 50) , rabbit polyclonal anti-LktA (1 in 30), or monoclonal antibodies to various RTX toxins (1 in 20) with *P. haemolytica* Ph2 culture supernate or active rLktA for 30 min at room temperature. The mixtures of antibody and toxin were then incubated with bovine neutrophils for 20 min before the neutrophils were stimulated with OZ. Column 1 shows the control CL response of neutrophils to OZ in the absence of LktA.

1: antibody (control, no LktA)

2: convalescent serum + LktA

3: polyclonal antibody + LktA

4: monoclonal antibody raised against rLktA + LktA

5: monoclonal antibody raised against *B. pertussis* AC toxin (9D4)+ LktA

6: monoclonal antibody 2-5 (IgG1) raised against AC toxin + LktA

7: monoclonal antibody raised against *A. pleuroneumoniae* toxin + LktA

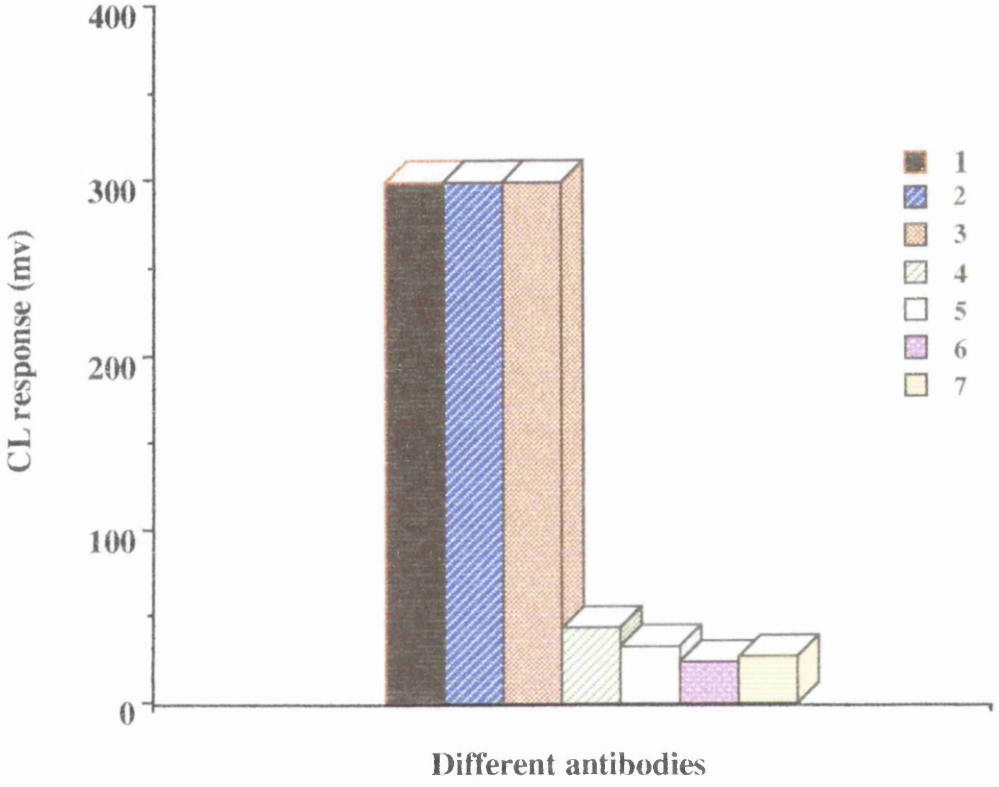

400
300
200
100
0
CL response (mv)
1
2
3
4
5
6
7
Different antibodies

Fig. 36.

Haemolytic activity of *P. haemolytica* for bovine and human blood.

The haemolytic activity of *P. haemolytica* isolates was determined on sandwich blood agar containing bovine (a) or human blood (b). Different strains of *P. haemolytica* were growm on BHIA containing 5% of either sheep or human blood overnight at 37 °C.

P. haemolytica strains

A: Ph62

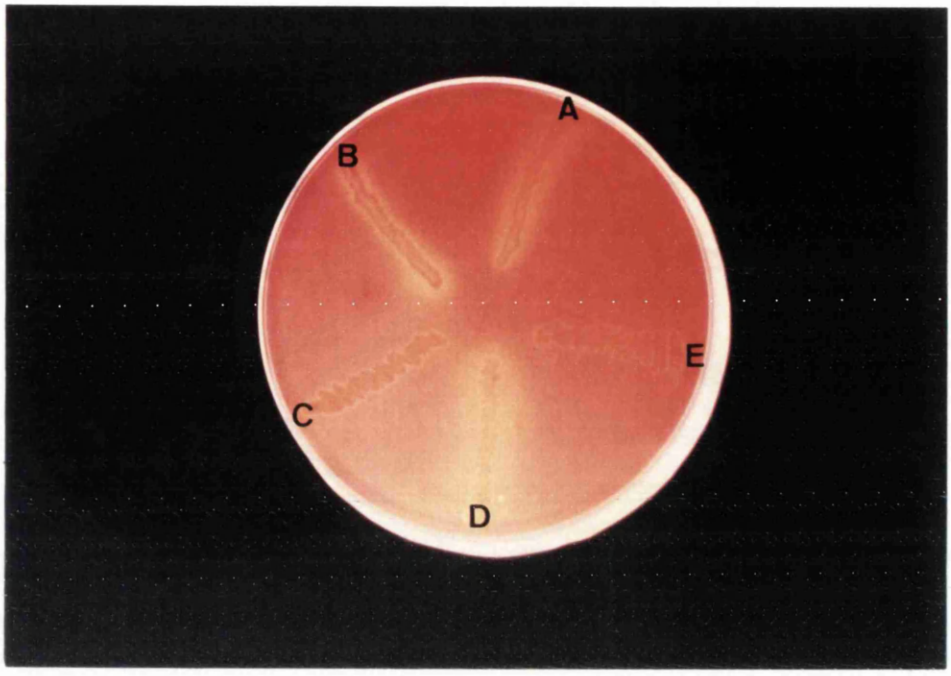
B: UT2

C: Ph140

D: FT4

E: Ph64

a



b

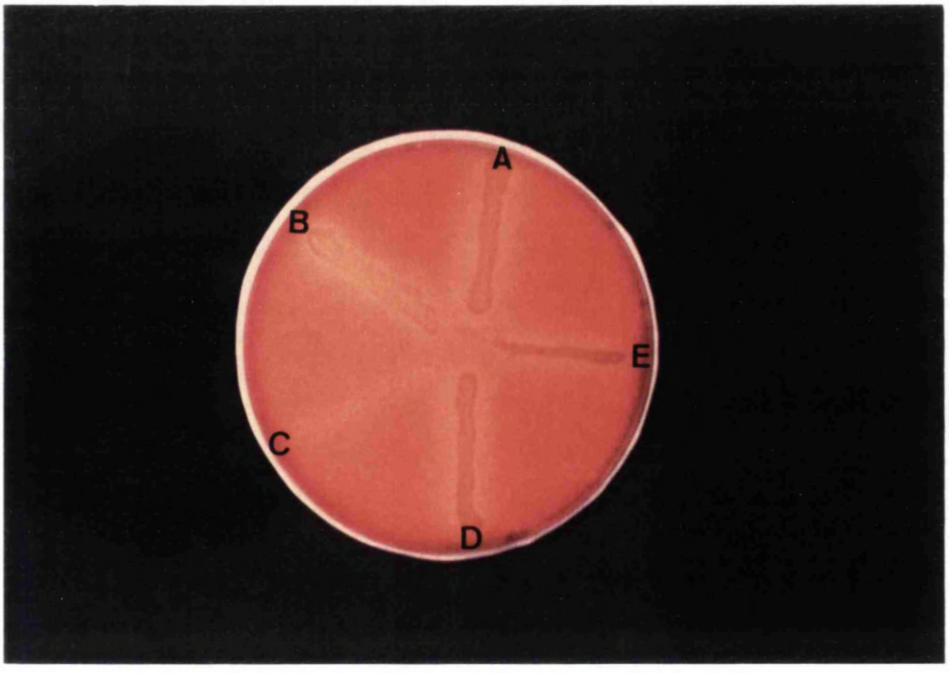


Fig. 37.

Haemolytic activity of *P. haemolytica* on BHIA.

The haemolytic activity of *P. haemolytica* Ph2 was determined on BHIA plus 5% sheep blood in the presence 20 mM $CaCl_2$. Plates were incubated for overnight at 37 °C.

concentrations of calcium ($CaCl_2$) (1 mM, 5 mM, 10 mM, 20 mM, 50 mM) or iron ($FeCl_3$) (50 µM, 100 µM, 200 µM, 300 µM) in attempts to increase the haemolytic activity on plates. The best condition for showing haemolysis was with 20 mM calcium chloride (fig. 37). Adding iron had little effect, presumably since BHIA is an iron-rich medium.

Even under the optimal conditions, the haemolytic activity was still low and not suitable for a close comparison of different strains. For this reason a microplate haemolysin assay was developed. The haemolytic activity of different cell-free culture supernates or rLktA was determined in a microplate assay with ovine erythrocytes. Ovine RBCs were incubated with serial dilutions of culture supernates from different isolates of *P. haemolytica* or dilutions of rLktA overnight at 37 °C. Sterile BHIB and *P. multocida* culture supernate or diluted urea were used as negative controls and saponin 0.1% was used as a positive control. The percentage of released haemoglobin was measured by ELISA reader at 540 nm. Although *P. haemolytica* generally has low haemolytic activity as seen on blood agar plates, culture supernates of different strains had different activities in the microplate assay (table 4). The highest activity was found with strain NCTC 10636 (Ph56) whereas strain FT4 had low activity. Only Ph6 had no haemolytic activity. However, in no case was the % haemolytic activity greater than 50%.

The haemolytic activity of culture supernate of *P. haemolytica* or rLktA was abolished by heating (100 °C for 30 min).

The haemolytic activity of LktA was temperature dependent, in that toxin did not produce lysis after overnight incubation at 4 °C. However, the toxin was shown to be bound to the cells at 4 °C (fig. 38). A comparison of haemolytic activity and cell binding of various LktA samples is given in the next section.

Fig. 38.

Binding of native and recombinant LktA to ovine RBCs.

The ovine RBCs were incubated with culture supernate of *P. haemolytica* Ph2 or rLktA from *E. coli* DH5αF'IQ (pGW42). The RBCs were then lysed and membrane proteins were separated by SDS-PAGE then immunoblotted after transfer to nitrocellulose. Rabbit polyclonal antibody raised against rLktA was used to detect binding. Culture supernate (lane A4) or rLktA (lane 1B) were used as positive controls and RBCs alone (lanes A5 and B2) were used as negative controls.

A native LktA

Lane 1: culture supernate of Ph2 with 1 mM $CaCl_2$ + ovine RBCs for 1 h at 37 °C

Lane 2: culture supernate of Ph2 with 1 mM $CaCl_2$+ ovine RBCs for 1 h at 4 °C

Lane 3: culture supernate of Ph2 with 1 mM EGTA + ovine RBCs for 1 h at 37 °C.

Lane 4: culture supernate of Ph2 (no RBCs, control)

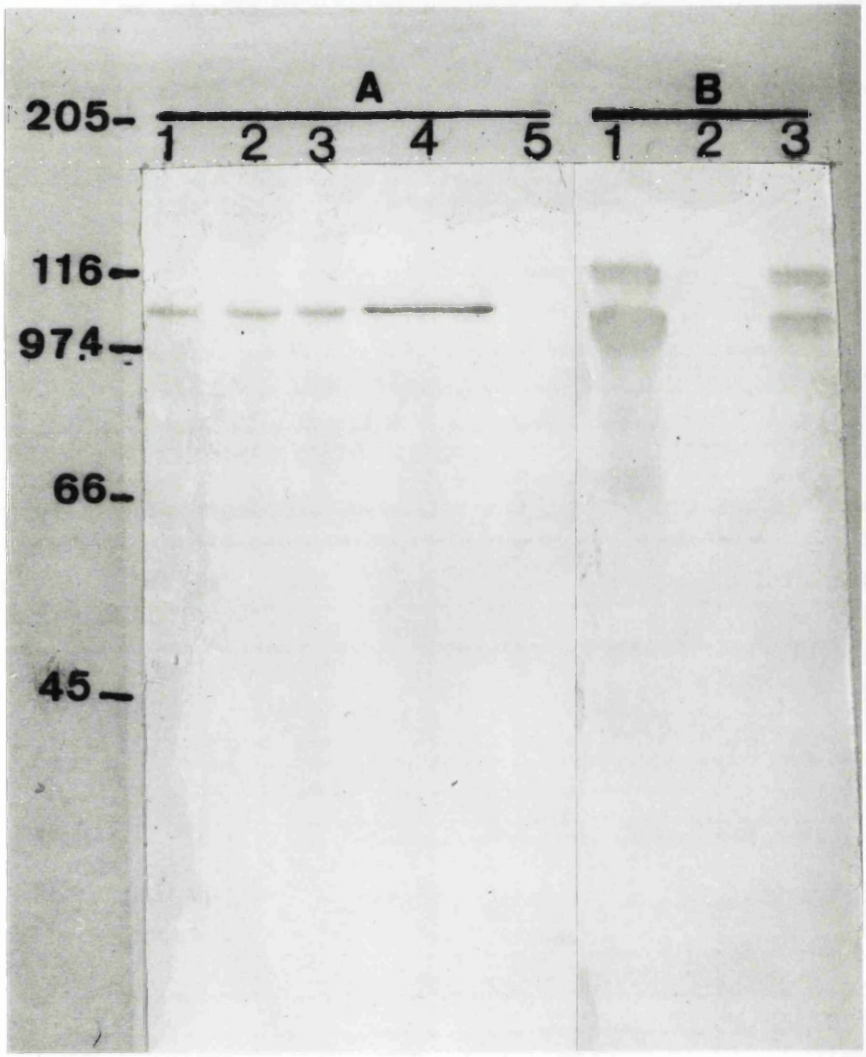
Lane 5: ovine RBCs alone (no LktA, control)

B rLktA

Lane 1: active rLktA alone (control)

Lane 2: ovine RBCs alone (control)

Lane 3: active rLktA bound to ovine RBCs


205-
116-
97.4-
66-
45-
A
1 2 3 4 5
B
1 2 3

3.13 BINDING OF LKT TO TARGET CELLS.

3.13.1 Binding of native and rLktA to ovine RBCs

In preliminary studies, sheep RBCs were incubated with native leukotoxin, from *P. haemolytica* strain Ph2, as well as with active rLktA from *E. coli* strain DH5αF'IQ containing pGW42 in order to determine ability of the toxins to bind to target cell membranes. Binding studies were done at 37 °C for 1 h in the presence of 1 mM calcium. The RBCs were incubated with BHIB or urea as controls. The RBCs were then lysed and membrane proteins were immunoblotted after transfer to nitrocellulose. Rabbit polyclonal antibody raised against rLktA was used to detect membrane-bound toxin. Fig. 38 shows the native LktA from *P. haemolytica* Ph2 (lane A1) and rLktA (lane B3) from *E. coli* was bound to the RBCs after 1 h at 37 °C. Native LktA was also shown to bind at 4 °C (lane A2) and in the presence of 1 M EGTA (lane A3) at 37 °C.

3.13.2 Binding of rLktA to various ruminant cells

Bovine neutrophils or mononuclear cells which had been separated from red blood cells were incubated with active and inactive rLktA at different temperatures (4 or 37 °C) for 1 h. The toxin band was detected by transferring the membrane proteins to nitrocellulose and probing with monoclonal antibody raised against rLktA. Fig. 39 shows the active (lane 2) and inactive (lane 3) rLktA bound to bovine RBCs at 37 °C and at 4 °C (lane 4, inactive; lane 5, active) and RBCs alone were used as control (lane 1). The inactive and active rLktA was also incubated with mononuclear cells for 1 h at 37 °C and 4 °C and again both forms were shown to bind (e.g. lanes 7 and 8, results for 37 °C). Similarly the rLktA (inactive lane 10) bound to bovine neutrophils at 37 °C and active was bound at 4 °C (lane 12) as well as 37 °C. In the leukocyte membranes preparation, the anti-rLktA reacted with a

Fig. 39.

Binding of rLktA to ruminant cells.

Bovine neutrophils or mononuclear cells which had been separated from red blood cells were incubated with active and inactive rLktA from *E. coli* SY327λ pir (pGW42) at different temperatures (4 or 37 °C) for 1 h. The toxin band was detected by transferring the membrane proteins to nitrocellulose and probed with monoclonal antibody raised against rLktA.

Lane 1: bovine RBCs (control)

Lane 2: bovine RBCs + active rLktA at 37 °C

Lane 3: bovine RBCs + inactive rLktA at 37 °C

Lane 4: bovine RBCs + inactive rLktA at 4 °C

Lane 5: bovine RBCs + active rLktA at 4 °C

Lane 6: bovine mononuclear cells (control)

Lane 7: bovine mononuclear cells + inactive rLktA at 37 °C

Lane 8: bovine mononuclear cells + active rLktA at 37 °C

Lane 9: bovine neutrophils (control)

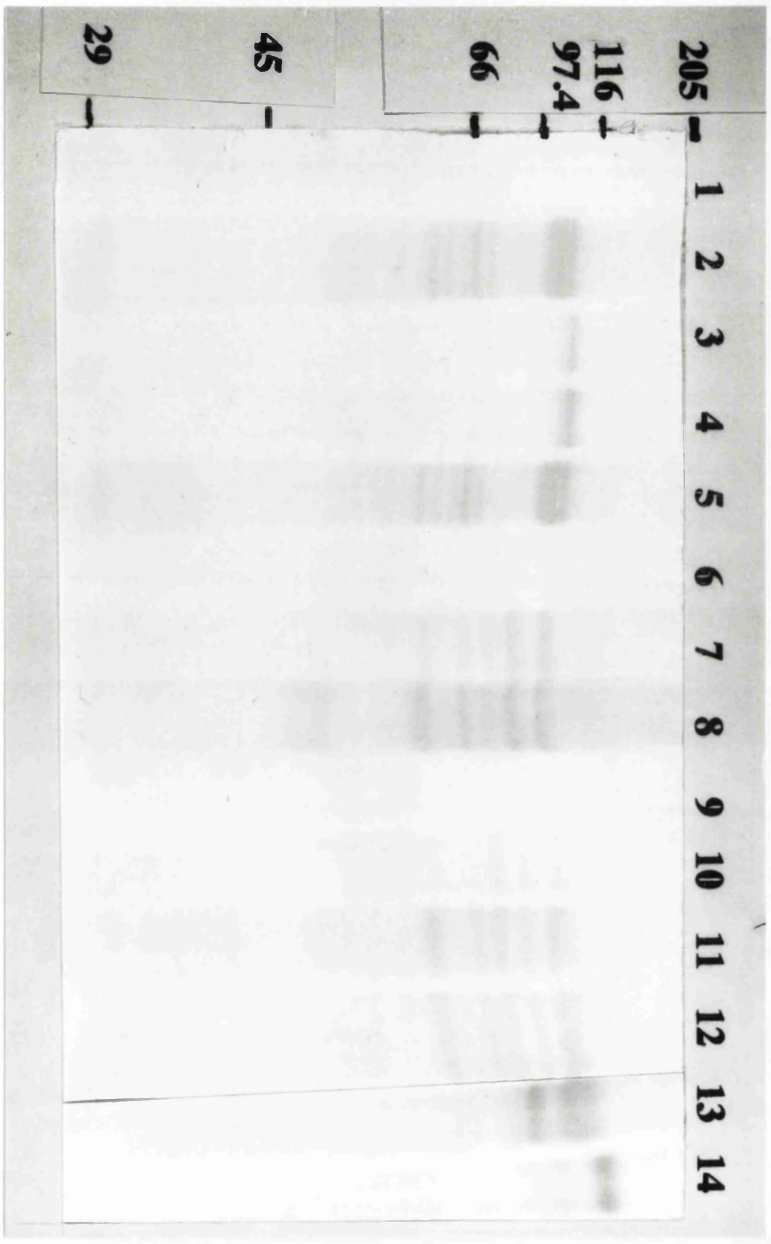
Lane 10: bovine neutrophils + inactive rLktA at 37 °C

Lane 11: bovine neutrophils + active LktA at 37 °C

Lane 12: bovine neutrophils + active rLktA at 4 °C

Lane 13: active rLktA (control)

Lane 14: inactive rLktA (control)


205
116
97.4
66
45
29
1 2 3 4 5 6 7 8 9 10 11 12 13 14

number of bands of <105 kDa indicating that LktA bound by leukocytes was degraded, presumably due to leukocyte proteases. The active rLktA (lane 13) and inactive rLktA (lane 14) were used as controls.

Although some of the native LktAs have low haemolytic or leukotoxin activities, they still bound to the target cells (data not shown). In addition, the inactive form of rLktA was bound to RBCs (fig 39, lane 3), but it had no detectable haemolytic activity. This result indicates that modification of LktA by LktC was important for lysis of RBCs but not for binding to RBCs.

3.13.3 Binding of LktA to human cells

To investigate the effect of LktA on non-ruminant cells, human leukocytes were separated from erythrocytes and then incubated with LktA. The activity of LktA on non-ruminant leukocytes was also measured by the CL inhibition assay (section, 3.10.2). Fig 40 shows faint bands indicating that both the active (lane 13) and inactive (lane 14) forms of rLktA were bound to some extent by human RBCs. The rLktA (active and inactive) was also bound to some extent by human mononuclear cells (lane 10 and 11, respectively) as well as to human neutrophils (lane 7 and 8). After incubation of human neutrophils with rLktA for 1 h , the neutrophils were separated and supernate was used for evaluation of unbound toxin by resolving the supernate by 10 % SDS-PAGE and then transferred to nitrocellulose and probed by monoclonal antibody raised against rLktA. Unbound protein of active (lane 5) and inactive (lane 6) form of rLktA were found. The same results were found with rabbit blood cells (data not shown).

3.13.4 Binding kinetics assessed by ELISA of unbound LktA

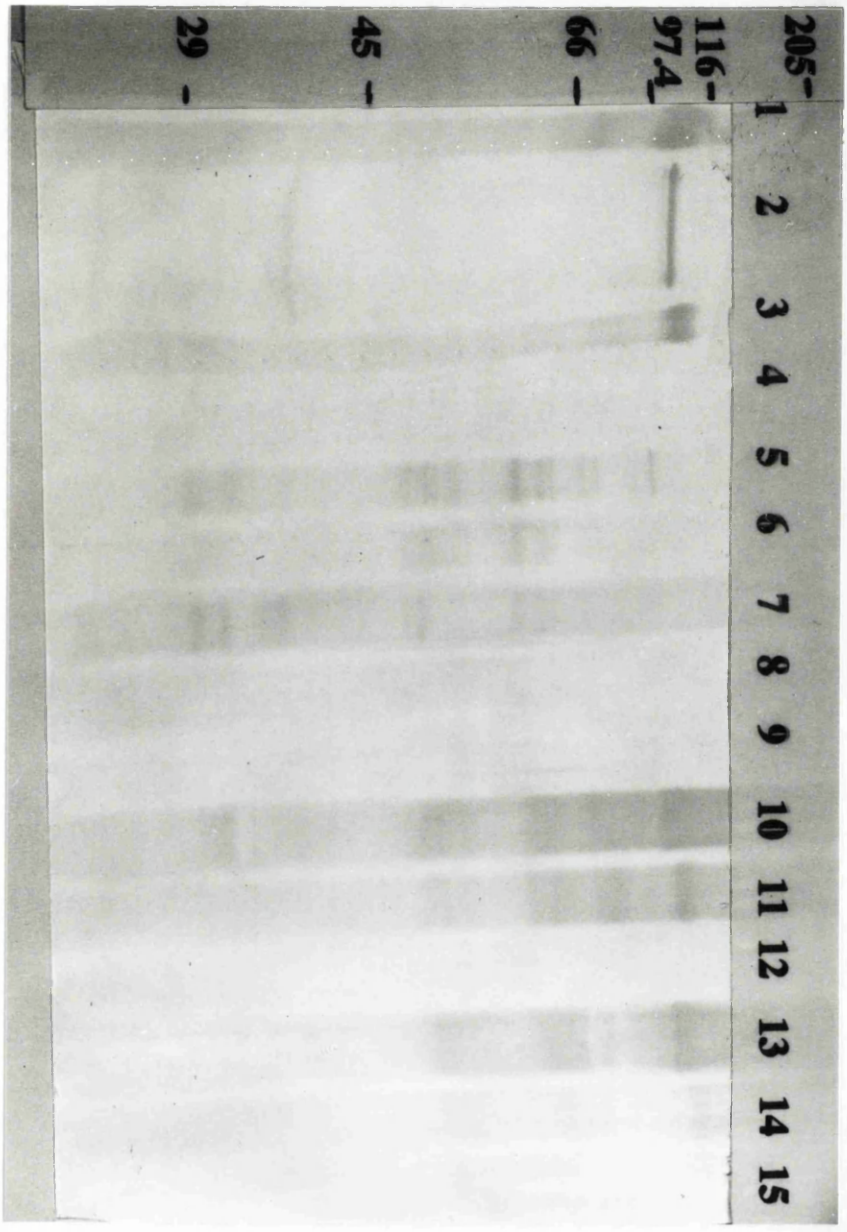
Bovine RBCs were incubated with native or rLktA toxin at different temperatures. The mixture was then centrifuged for 15 min at 15,000 x g and

Fig. 40.

Binding of LktA to human cells.

Human neutrophils, mononuclear cells or red blood cells were incubated with active or inactive rLktA at 37 °C for 1 h. The toxin band was detected by transferring the membrane proteins to nitrocellulose and probed with monoclonal antibody raised against rLktA. The RBCs, mononuclear cells, neutrophils, native LktA, active rLktA and inactive rLktA were used as controls.

Lane 1: active rLktA (from *E. coli* SY327λ pir (pGW42)(control)
Lane 2: native LktA from Ph2 culture supernate (control)
Lane 3: inactive rLktA from *E. coli* SY327λ pir (pGW64)(control)
Lane 4: rLktC from *E. coli* SY327λ pir (pGW78)(control)
Lane 5: supernate of active rLktA + neutrophils
Lane 6: supernate of inactive rLktA + neutrophils
Lane 7: active rLktA + neutrophils
Lane 8: inactive rLktA + neutrophils
Lane 9: neutrophils (control)
Lane 10: active rLktA + mononuclear cells
Lane 11: inactive LktA + mononuclear cells
Lane 12: mononuclear cells (control)
Lane 13: active rLktA + RBCs
Lane 14: inactive rLktA + RBCs
Lane 15: RBCs (control)


205-
116-
97.4
66 -
45 -
29 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

the supernate was used for evaluation of unbound toxin by ELISA. A reduced level of toxin remaining in the supernate showed that the toxin was bound to RBCs at 4 °C as well as at 37 °C and when the two different temperatures were compared, more toxin was bound to the RBCs at 37 °C than at 4 °C (fig. 41).

The results with bovine mononuclear cells in the binding assay are shown in fig. 42. The mononuclear cells were incubated with rLktA for 3, 5, 10 and 60 min at 4 °C or at 37 °C and then unbound toxin was measured by ELISA. More toxin was bound at 37 °C for 3 min than at 4 °C for 1 h (fig. 42).

With bovine neutrophils, similar results were obtained. As fig. 43 shows, the binding of rLktA at 37 °C was quicker than at 4 °C.

These ELISA results supported the findings of the previous investigation where binding was assessed by immunoblotting.

3.13.5 Binding assays in presence of protease inhibitors

In binding assays with some target cell types e.g. neutrophils, the bound toxin appeared to be degraded even at 4 °C i.e. only low mol. wt (<105 kDa) bands were seen by immunoblotting of membrane fractions with antibodies specific for LktA (figs. 39 and 41). Thus, protease inhibitors were investigated in an attempt to improve the resolution of the assay. The stability of urea extract of rLktA in HH and native LktA in BHIB was examined in the presence and absence of a cocktail comp-rising: serine and thiol protease inhibitor, leupeptin; a serine protease inhibitor, PMSF; an acidic protease inhibitor, pepstatin; and a metalloprotease inhibitor, EDTA (Boehringer-Mannheim, Biochemical Information Leaflet, May 1987). The cocktail was added to bovine and human neutrophils and incubated for 30 min at room temperature before adding the LktA preparation. The mixture were then

Fig. 41.

Measurement by ELISA of unbound LktA in binding assays with bovine RBCs.

The amount of unbound toxin was evaluated by ELISA. The RBCs were incubated with the active form of rLktA from *E. coli* SY327λ pir (pGW42) for 3, 5 and 10 min at room temperature or 3 and 5 min at 4 °C. The mixture was centifuged for 15 min at 15,000 x g and supernate was used for evaluation of unbound toxin by ELISA.

1: control rLktA (no RBCs).

2: rLktA after incubation with RBCs for 3 min at 37 °C.

3: rLktA after incubation with RBCs for 5 min at 37 °C.

4: rLktA after incubation with RBCs for 10 min at 37 °C.

5: control rLktA (no RBCs).

6: rLktA after incubation with RBCs for 3 min at 4 °C.

7: rLktA after incubation with RBCs for 5 min at 4 °C.

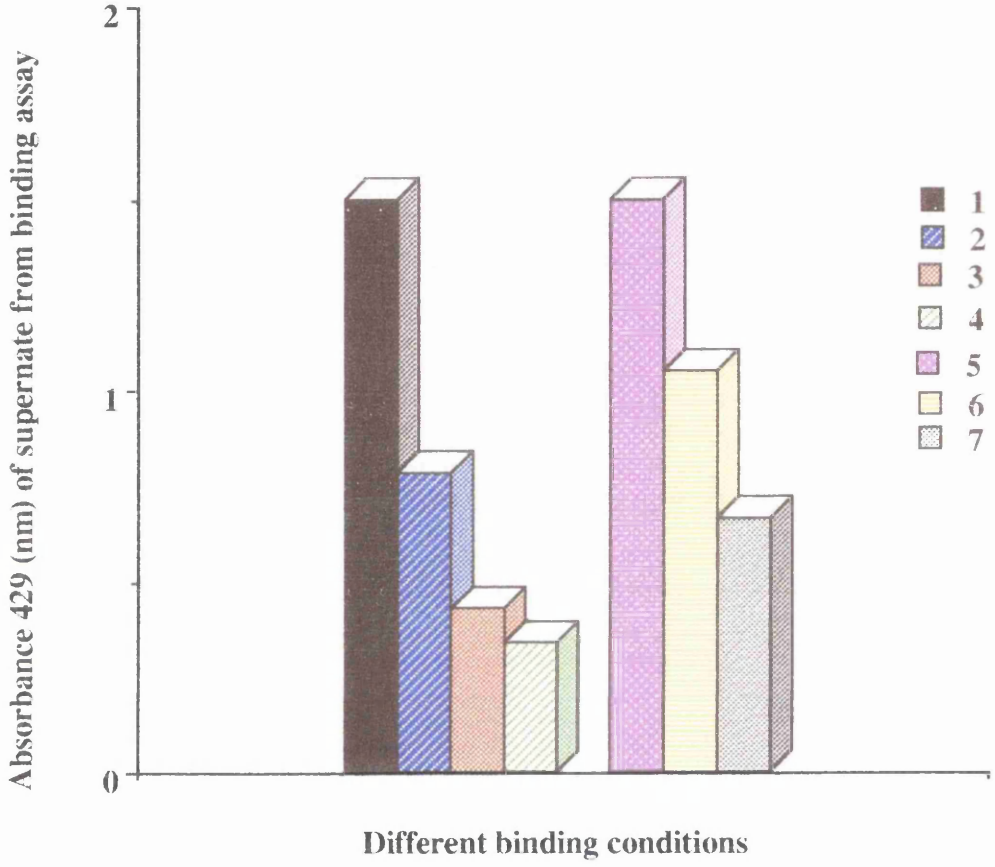

Absorbance 429 (nm) of supernate from binding assay
2
1
0
1
2
3
4
5
6
7
Different binding conditions

Fig. 42.

Measurement by ELISA of unbound LktA in binding assays with bovine mononuclear cells.

The unbound rLktA was measured by ELISA. The mononuclear cells were incubated with active rLktA from *E. coli* SY327λ pir (pGW42) at 3, 5, 10 and 60 min at 4 °C or at 37 °C and the supernate was separated as described in fig. 41 and used for evaluation of unbound toxin.

1: control rLktA (without mononuclear cells).

2: rLktA after incubation with mononuclear cells for 3 min at 4 °C.

3: rLktA after incubation with mononuclear cells for 5 min at 4 °C.

4: rLktA after incubation with mononuclear cells for 10 min at 4 °C.

5: rLktA after incubation with mononuclear cells for 60 min at 4 °C.

6: control rLktA (without mononuclear cells)

7: rLktA after incubation with mononuclear cells for 3 min at 37 °C.

8: rLktA after incubation with mononuclear cells for 5 min at 37 °C.

9: rLktA after incubation with mononuclear cells for 10 min at 37 °C

10: rLktA after incubation with mononuclear cells for 60 min at 37 °C

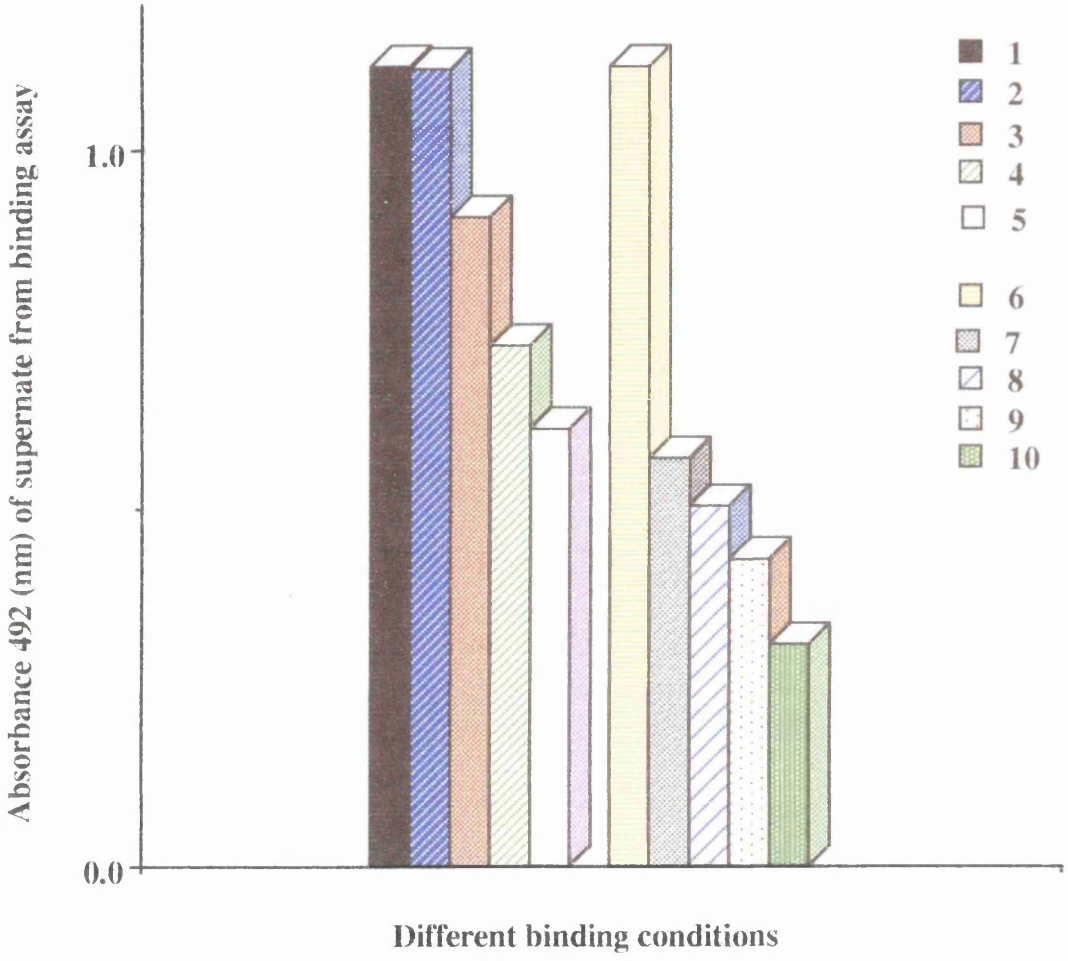

Absorbance 492 (nm) of supernate from binding assay
1.0
0.0
1
2
3
4
5
6
7
8
9
10
Different binding conditions

Fig. 43.

Measurement by ELISA of unbound LktA in binding assays with bovine neutrophils.

The unbound rLktA with bovine neutrophils in binding assay also was measured by ELISA. The neutrophils were incubated with rLktA for 3, 5 and 10 min at 4 °C or at 37 °C and then supernate was separated and used for ELISA. The binding of rLktA at 37 °C was quicker than at 4 °C.

1: control rLktA (no neutrophils).

2: rLktA was incubated with neutrophils for 3 min at 37 °C.

3: rLktA was incubated with neutrophils for 5 min at 37 °C.

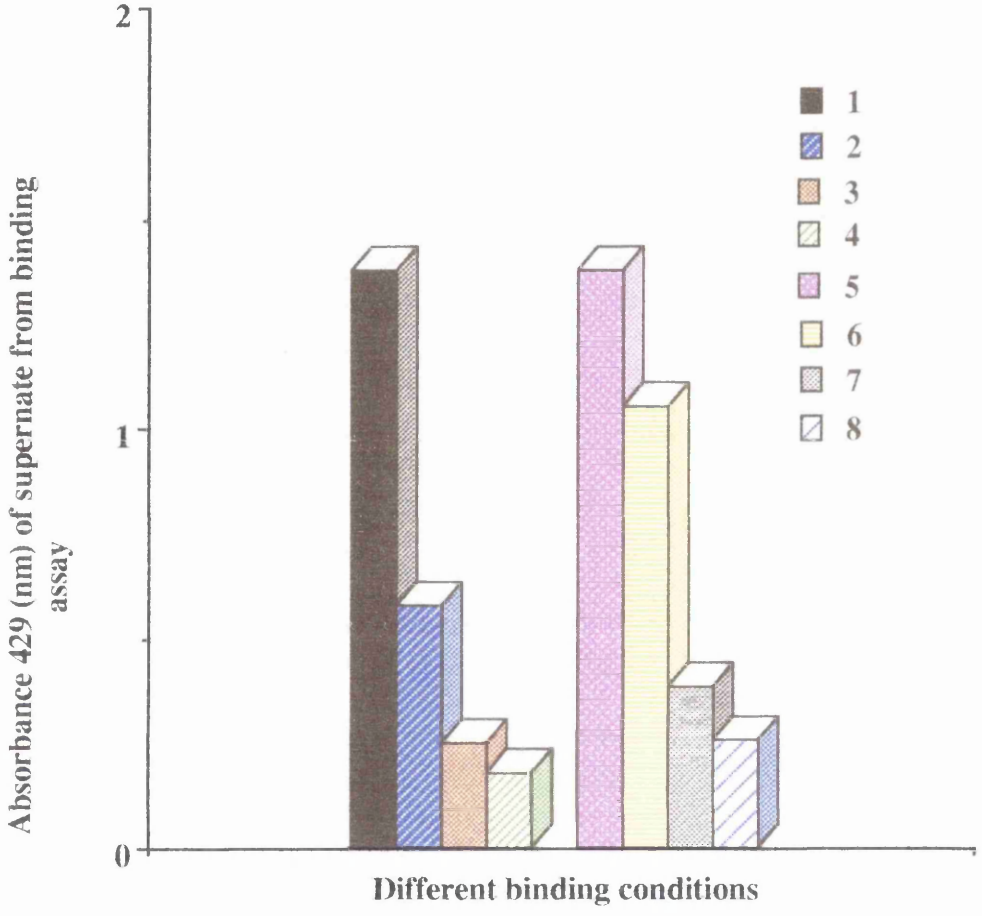
4: rLktA was incubated with neutrophils for 10 min at 37 °C.

5: control rLktA (no neutrophils).

6: rLktA was incubated with neutrophils for 3 min at 4 °C.

7: rLktA was incubated with neutrophils for 5 min at 4 °C.

8: rLktA was incubated with neutrophils for 10 min at 4 °C.


2
1
0
Absorbance 429 (nm) of supernate from binding assay
1
2
3
4
5
6
7
8
Different binding conditions

incubated at 37 °C for 1 h. The cells were then lysed, washed and the membrane proteins were resolved by SDS-PAGE and immunoblotted after transfer to nitrocellulose. Polyclonal antibody raised against rLktA was used to detect bound toxin. The cocktail of protease inhibitors did not appear to inhibit the toxin degradation.

3.14 CAMP test

The haemolytic activity of some RTX toxins has been reported to be enhanced in the presence of *Staphylococcus aureus* β-toxin (Jansen *et al.*, 1995). In this investigation, the haemolytic and non-haemolytic strains of *P. haemolytica* were compared in the CAMP test.

All strains of *P. haemolytica* used in this study were grown on BHIA containing 5% sheep blood for 24-48 h at 37 °C in air plus 10 % CO_2 and tested against a β-toxin-producing *Staphylococcus aureus*. A group B *Streptococcus* was used as positive control (fig. 44).

Analysis of all *P. haemolytica* serotype reference strains revealed that all serotypes produce a co-haemolytic CAMP effect although with unequal intensity. Fig. 44 shows the results with 7 strains of *P. haemolytica* (5 biotype A, 1 biotype T, 1 untypable strain) and 1 strain of *Streptococcus* tested against *Staphylococcus aureus*. The biggest co-haemolytic zone was found with strain UT3 (untypable, No. 6). No obvious differences were found between the two biotypes or the various serotypes. Only one strain of A1 (Ph6) did not produce a co-haemolytic CAMP effect (No. 7).

3.15 Measurement of chemotaxis

The micropore filter assay is a simple way of observing the reactions of neutrophils to chemotactic factors in vitro. It is essentially a two chamber

Fig. 44.

The synergistic haemolytic effect of *P. haemolytica* and *Staphylococcus aureus* (CAMP test)

Five strains of *P. haemolytica* were grown on BHIA containing 5% sheep blood for 24 h at 37 °C in air plus 10 % CO_2 and tested for haemolysis against a β-toxin-producing *Staphylococcus aureus*. A group B *Streptococcus* was used as CAMP positive control. Analysis of all *P. haemolytica* serotype reference strains revealed that all serotypes (except Ph6) produce a co-haemolytic CAMP effect, although with unequal intensity.

1: *Staphylococcus aureus.*

2: group B *Streptococcus*

3: *P. haemolytica* Ph2

4: *P. haemolytica* Ph42

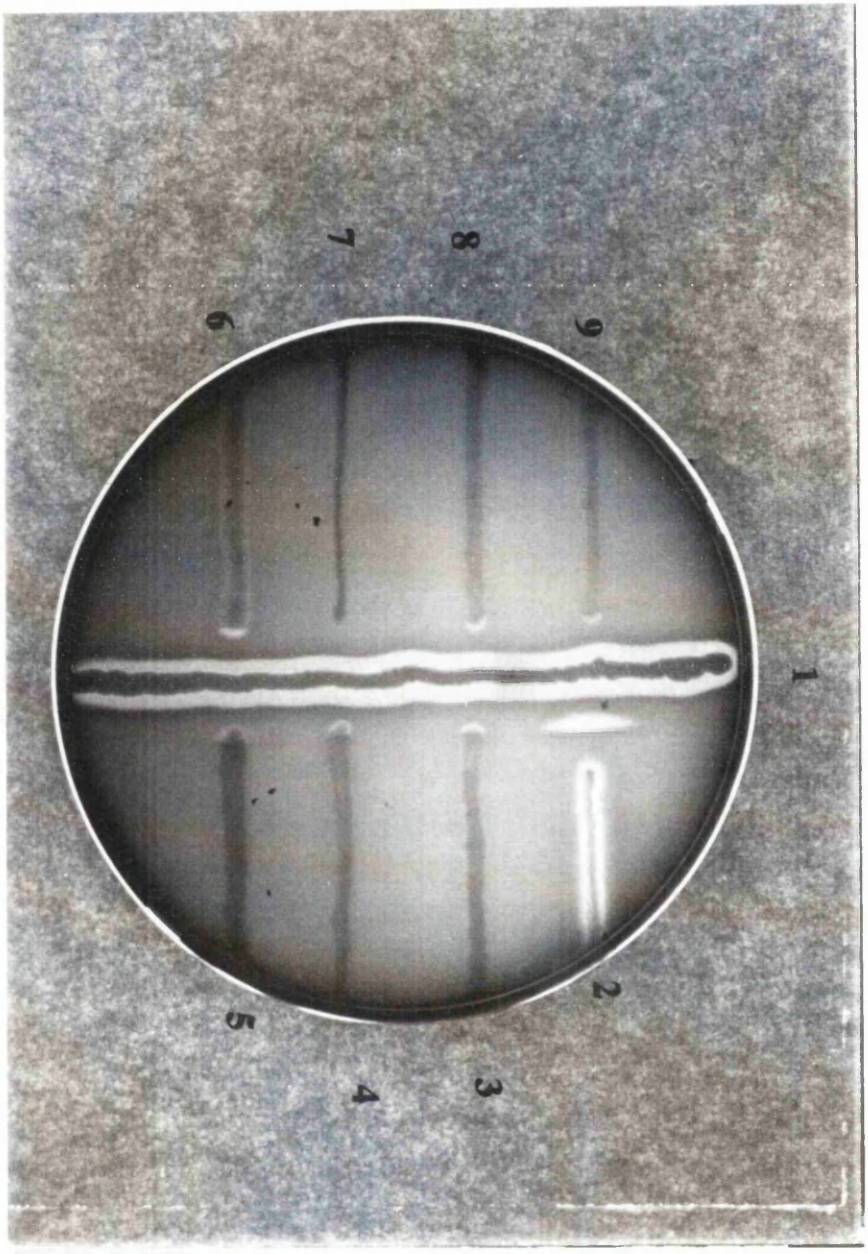
5: *P. haemolytica* Ph58

6: *P. haemolytica* UT3

7: *P. haemolytica* Ph6

8: *P. haemolytica* Ph70

9: *P. haemolytica* Ph60


7
8
9
6
1
2
3
4
5

apparatus in which neutrophils are placed in the upper chamber and separated by a 5 μm filter from the chemotactic factor in the lower chamber. In order to investigate the effect of LktA on the chemotactic response of neutrophils two assays were set up: the neutrophils, with or without toxin, were placed in the upper chamber and OZ as the chemotactic factor was placed in the lower chamber or neutrophils alone were placed in the upper chamber and different concentrations of LktA or purified LPS were placed in the lower chamber to see if either had any chemotactic activity. The chamber was then incubated for 45 min at 37 °C in 10 % CO_2 and the filter was then fixed and stained. The migrated cells on the underside of the filter were counted. As table 7 shows, culture supernate, rLktA (active and non-activate) and purified LPS had chemotactic activity to different extents. The highest chemotactic activity was found with culture supernate of Ph2. There was no significant difference in chemotactic activity between active or non-activated rLktA. LPS of 40 μg/ml had chemotactic activity although this activity was abolished with high concentrations of LPS (4 mg/ml) (table 7).

3.16 EFFECT OF LKT ON LEUKOCYTE MORPHOLOGY AND MOVEMENT

3.16.1 Effect of LktA on bovine leukocytes

The above CL-inhibition assays showed that leukotoxin binds to and affects the respiratory burst of both ruminant and human neutrophils. The effect of LktA on leukocyte morphology and movement was investigated in a cell tracking assay. Purified leukocytes (containing 85% neutrophils and 15% monocytes and lymphocytes) were placed in a filming chamber along with different concentrations of active or inactive rLktA and native leukotoxin. None of the LktA preparations had any significant effect on

Table 7.

Chemotactic response of bovine neutrophils to different preparations of LktA

Chemotactic response of bovine neutrophils to different preparation of LktA from culture supernates of *P. haemolytica* (29 µl), rLktA from *E. coli* (29 µl of 1 in 5000 dilution) and LPS (40 µg/ml).

	Chemotactic response of bovine neutrophils (Average number of migrated cells per field)*
Negative control	19.5
positive control (OZ)	89
Ph2	202.4
Ph26	82.6
Ph42	50.6
Ph48	32.5
Ph54	37
Ph58	202
Ph60	51.6
Ph250	56
LPS (40 µg/ml)	105
Active rLktA	200
non-active rLktA	180

*mean of three separate experiments

movement of the bovine neutrophils. The effect of LktA on bovine neutrophil morphology is shown in fig. 45a, b, c, d and e. Fig. 45b shows the target cells mixed with active rLktA for 10 sec at 37 °C, before any effect was noticed. After 2 min the toxin had caused cell swelling and some of cells were lysed (fig. 45c). After 5 min, more than 50% of the cells were lysed (fig. 45d). All the cells were killed by active rLktA in less than 15 min by the process of cell swelling and lysis (fig. 45e). However during this time inactive rLktA at the same protein concentration had no effect on bovine leukocytes (fig. 45a) and these were identical to untreated controls. Native LktA had a similar effect to that of active rLktA (not shown). The rLktA also killed monocytes and neutrophils but it had no effect on lymphocytes as these are the only cells surviving after 15 min (fig. 45e).

3.16.2 Effect of LktA on human leukocytes

Human leukocytes were separated as above and then treated with active or inactive rLktA. The leukotoxin did not kill the cells. However, the toxin caused the migration of these cells (fig. 46). The speed of the neutrophils was observed by displacement of randomly moving objects determined by the least squares approximation in a measured time interval which gives an apparent speed that approaches the true speed as the measuring interval decreases. It was reported that the use of 5 second time intervals gave considerably higher cell speeds than did a 10 second intervals (Chettibi *et al.*, 1994). With active rLktA, the human neutrophils appeared to move with high speed, and with persistence and a high diffusion coefficient. This contrasted with non-active rLktA where speed was 1/2 that with the active toxin and there was no persistence or diffusion coefficient. This indicates that with active toxin, cells appear to move in the same direction for sometime before changing direction. This may be due to a lack of adhesion to

Fig. 45.

Effect of LktA on bovine leukocyte morphology and movement.

To show the effect of LktA on bovine leukocytes, the leukocytes were separated from RBCs with Percoll and most of monocytes and lymphocytes were removed with Histopaque. The purity of neutrophils was 85 % and most of the remaining cells were lymphocytes. The cells were treated with 1 µg/ml final concentration of active rLktA from *E. coli* SY327λ pir (pGW42) or the inactive form of rLktA from *E. coli* SY327λ pir (pGW64) in a filming chamber at 37 °C.

a: neutrophils incubated with inactive rLktA after 20 min

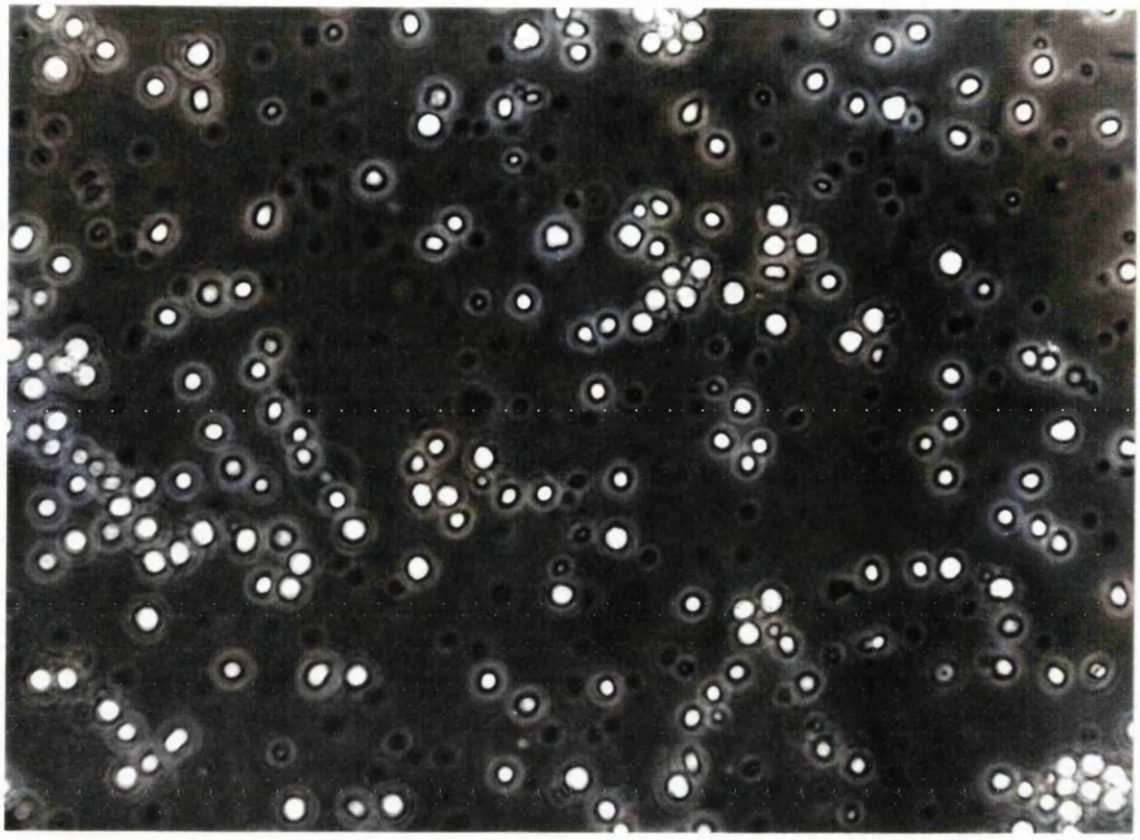
b: neutrophils incubated with active rLktA after 10 sec

c: neutrophils incubated with active rLktA after 2 min

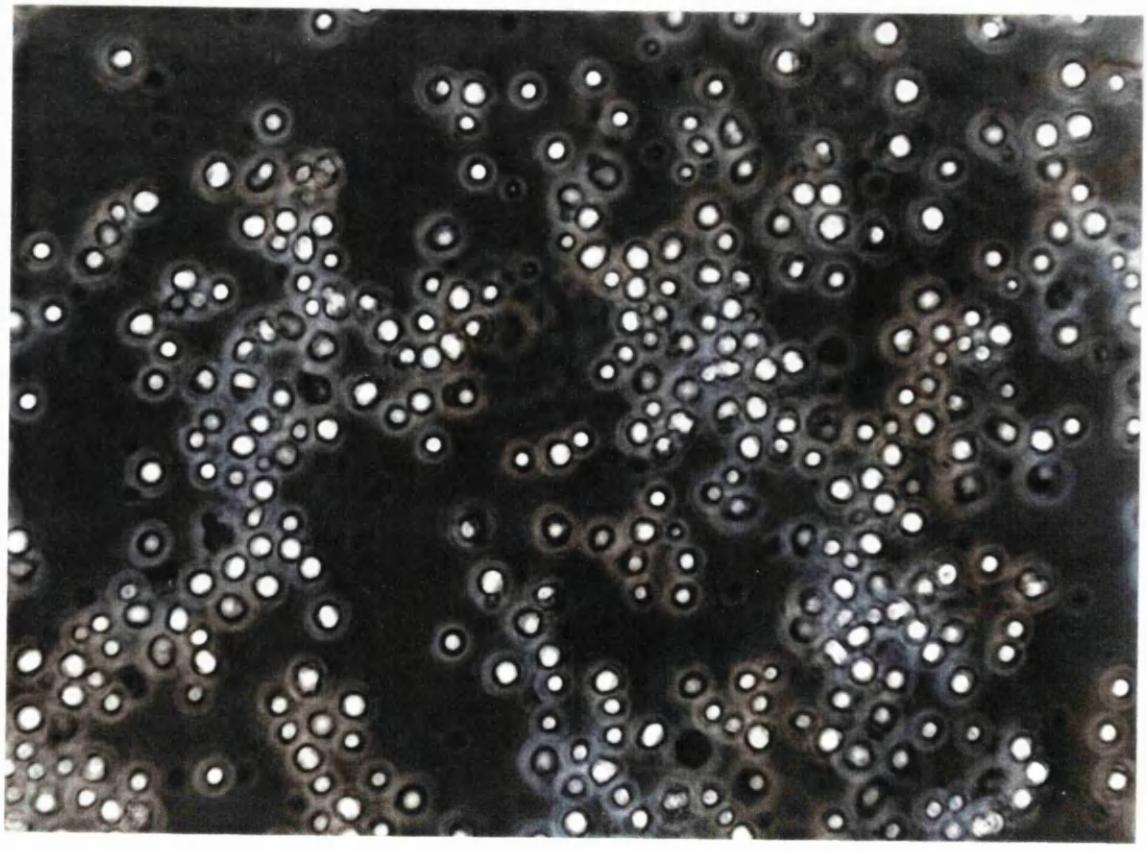
d: neutrophils incubated with active rLktA after 5 min

e: neutrophils incubated with active rLktA after 15 min

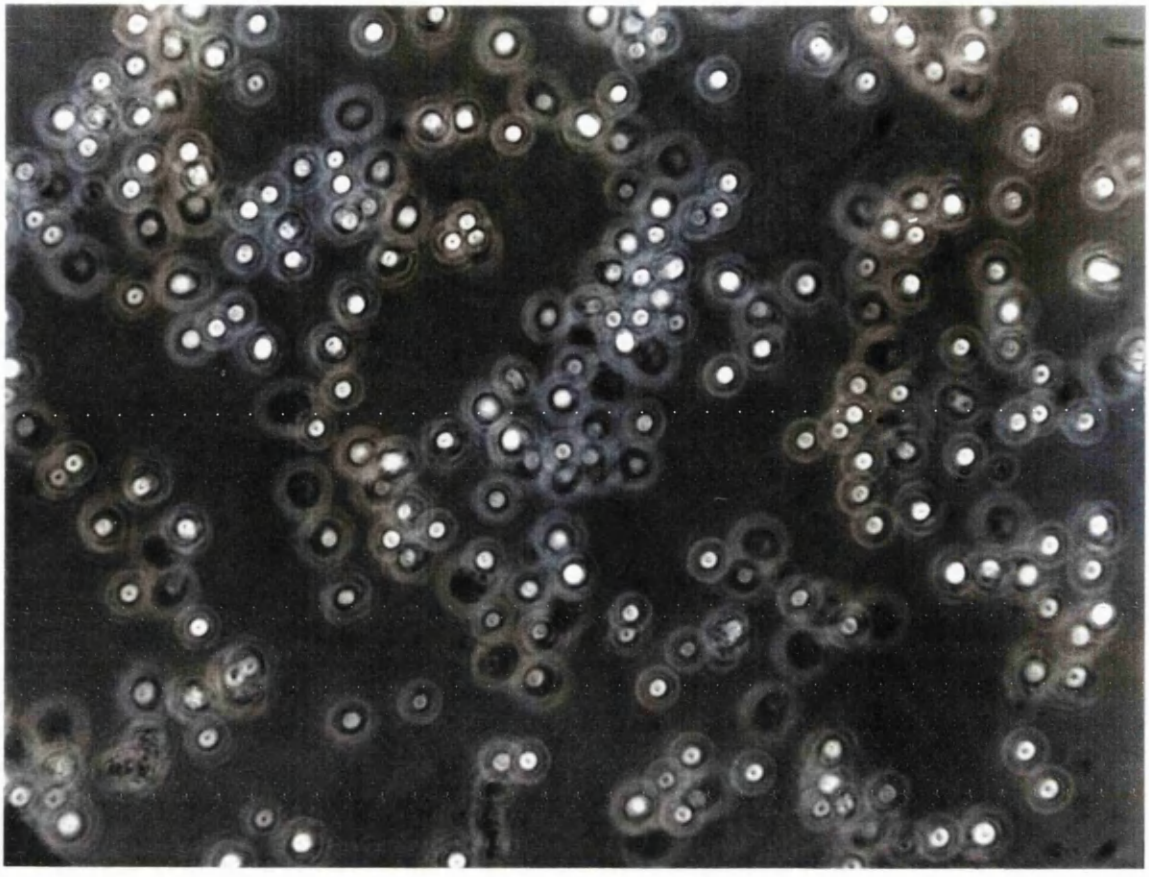
a



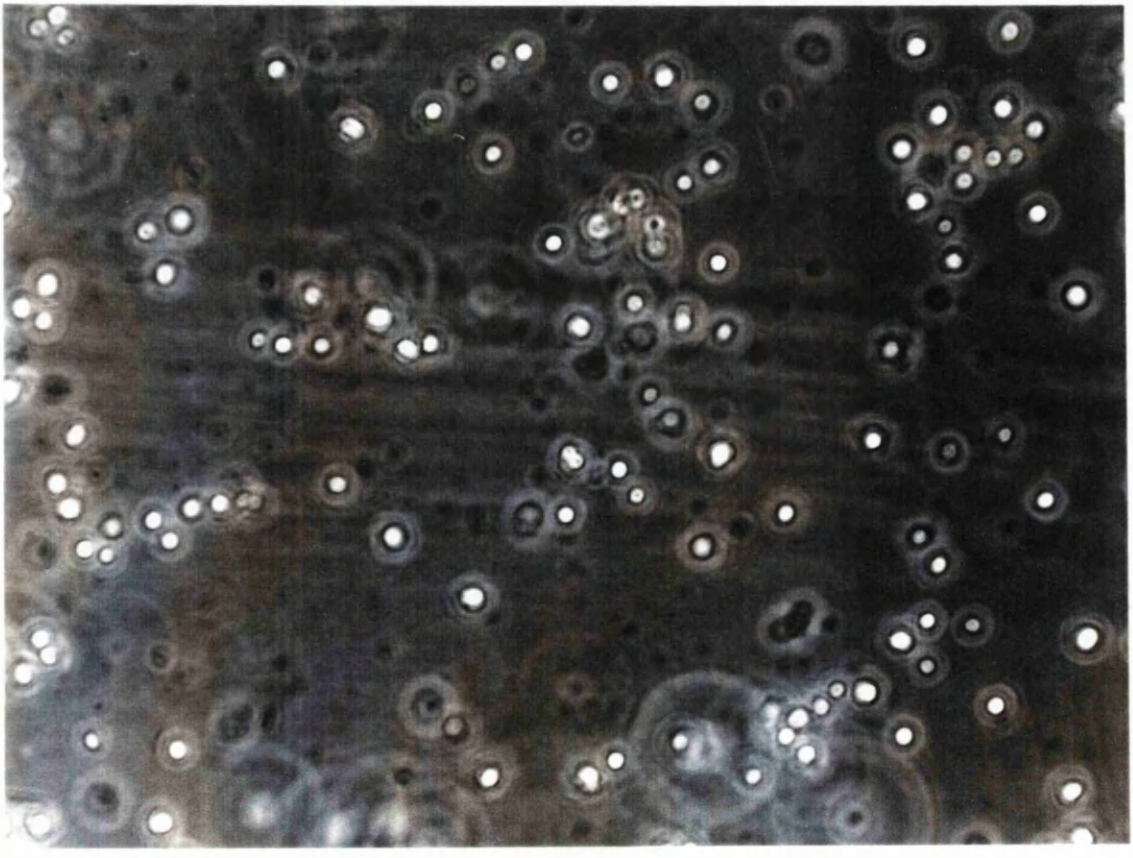
b



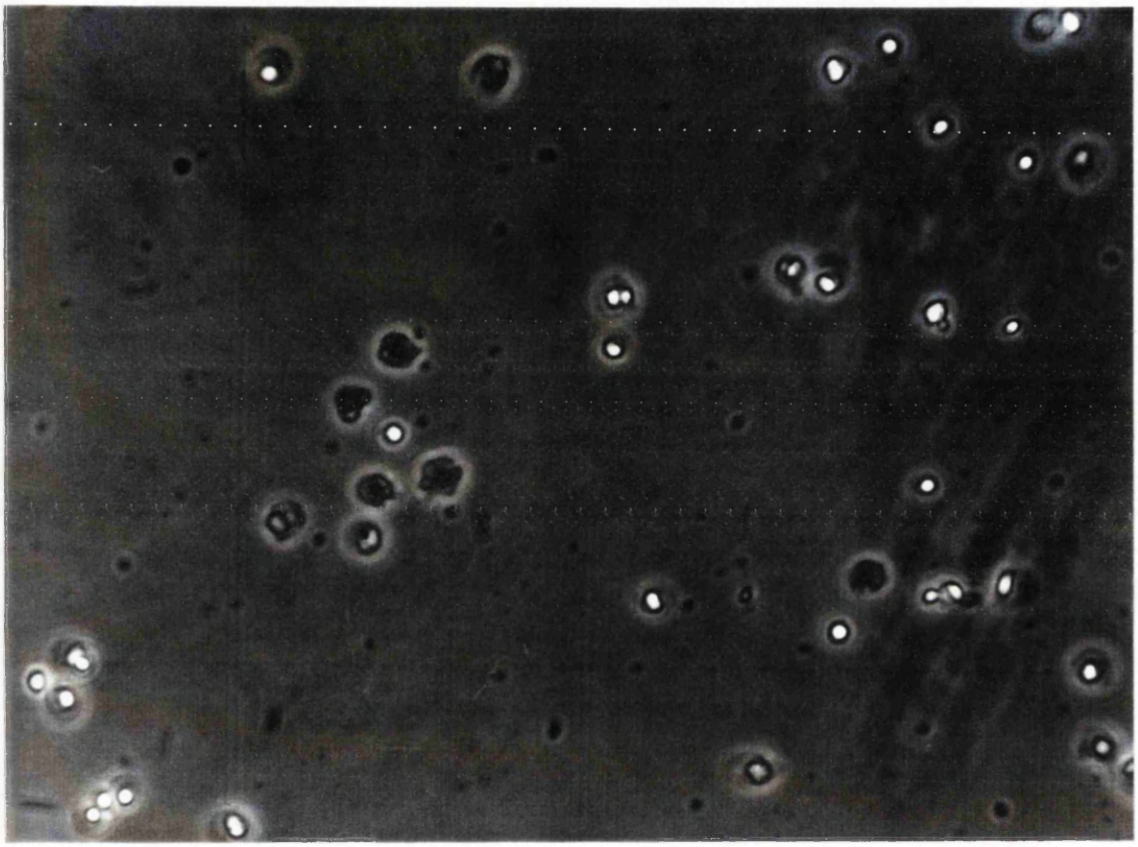
c



d



e



the substrate (albumin-coated) with active rLktA. With non-active rLktA, cells are moving and adhering and therefore were not able to displace from their original position (table 8).

3.17 LKT ACTIVATION IN VITRO

The inactive toxin was activated *in vitro* by addition of LktC and cytosolic activating factor (CAF), an *E. coli* cell extract. LktA was prepared from *E. coli* strain SY327λ pir containing pGW64 (encoding *lktA*) and LktC was prepared from *E. coli* strain SY327λ pir containing pGW78 (encoding *lktC*). Activated LktA killed the bovine neutrophils and inhibited the CL response but did not kill the rabbit neutrophils. The LktC and CAF or LktA were used as controls and had no effect on CL inhibition assay (table 4). Recombinant LktC alone did not activate rLktA, but in the presence of CAF, the inactive rLktA was activated (table 9).

Fig. 46.

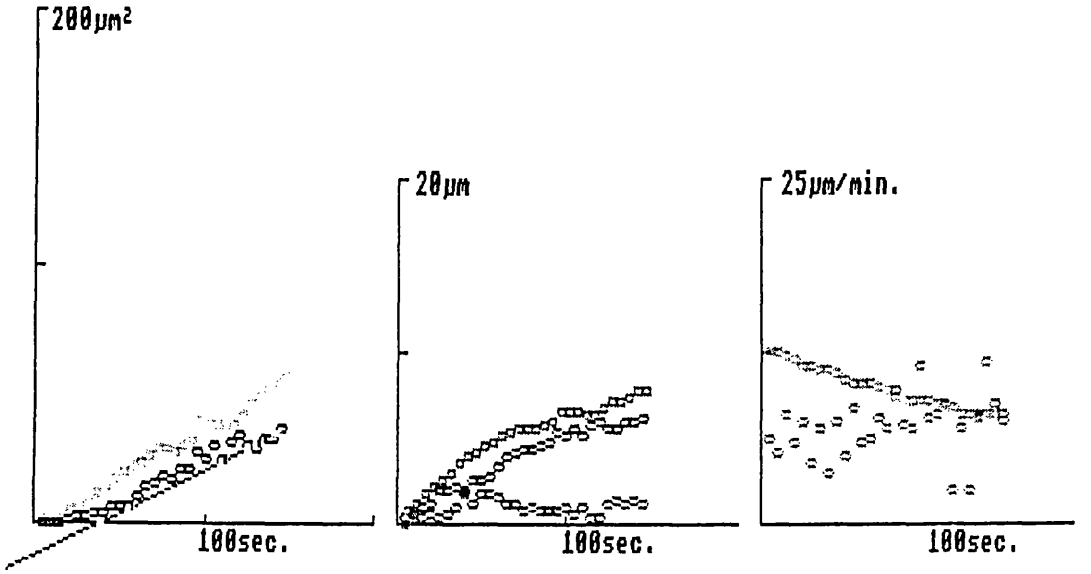
Effect of LktA on human leukocyte morphology and movement

Human leukocytes were separated as in fig. 45 and then treated with 300 µl (5 µg/ml) active or inactive rLktA. The migration of cells and the speed of neutrophils was observed by displacement of randomly moving objects determined by the least squares approximation in a measured time interval. This gives an apparent speed that approaches the true speed as the measuring interval decreases. Drift (a component of non-random locomotion) was calculated by determining the centre of cell density by carrying out a vectoral sum of the X and Y displacements of each cell centre to determine the average population displacement. Plots shown the mean accumulated displacement of neutrophils in response to active and inactive rLktA. Uncorrected data (•), drift-corrected data (▫). Straight lines drawn thought the linear part of the drift-corrected curved for active rLktA-treated cells and drift-corrected curve for inactive rLktA-treated cells gave persistence values of 38.51 seconds and -48 seconds, respectively.

1: 300 µl of active form of LktA

2: 300 µl of inactive form of rLktA

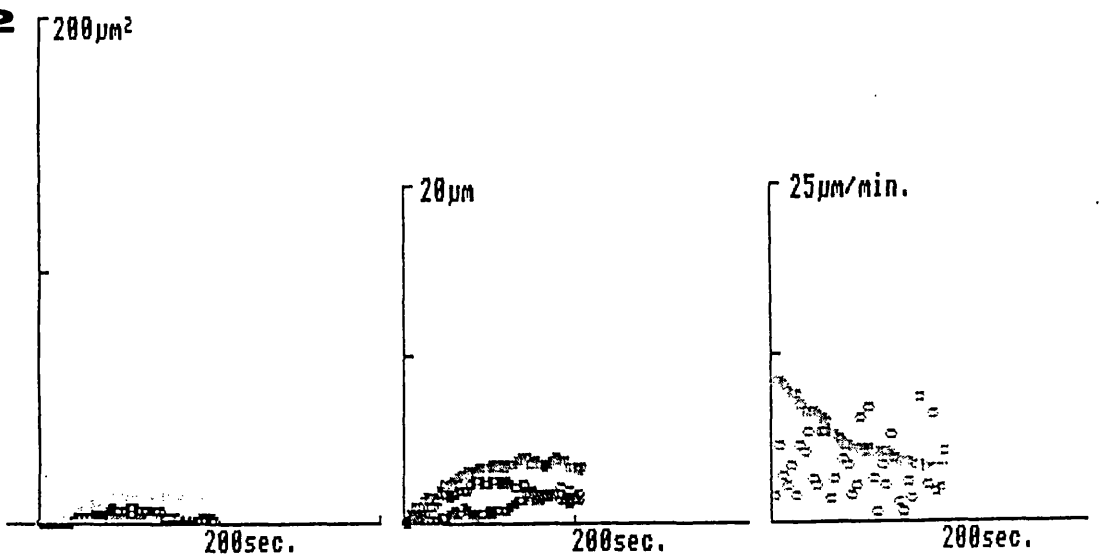
1



Diff. coeft 0.128µm²/sec.. Distance 6.38µm Speed 7.6µm/min
Persistance 38.51sec. Drift 0.98µm at -96° Cells 50-32

Area 29.5µm²-29.5µm²
Frames 30*5sec.

2



Diff. coeft 0.012µm²/sec. Distance 1.47µm Speed 4.6µm/min
Persistance -48.00sec. Drift 0.99µm at -119° Cells 42-16

Area 24.4µm²-17.9µm²
Frames 42*5sec.

Table. 8.

The diffusion coefficient, persistence and speed of locomotion of human neutrophils treated with active and inactive rLkt.

Human neutrophils were treated with 300 µl (5 µg/ml) of active and inactive rLkt.

treatment	diffusion coefficient ($\mu m^2/s$)	persistence (s)	speed (µm/min)
active rLkt	0.128	38.51	7.6
inactive rLkt	0.012	-48.00	4.6

Table 9.

Activation in vitro

The inactive toxin was activated by addition of LktC and CAF to LktA in vitro. LktA was prepared by growing the *E. coli* strain SY327λ pir containing plasmid pGW64 (encoding *lktA*) and the LktC was prepared by growing the *E. coli* strain SY327λ pir containing plasmid pGW78 (encoding *lktC*). For activation of inactive toxin, 50 µl of inactive form of rLktA was added to mixture of 50 µl of LktC and 50 µl of CAF and incubated for 30 min at 4 °C. The mixture was then added to bovine, human and rabbit neutrophils and incubated for further 20 min at 38 °C. The cells were stimulated with OZ. The activity of LktA was measured by CL inhibition assay. Activated LktA killed the bovine neutrophils and inhibited CL but did not kill the human or rabbit neutrophils. The LktC and CAF or LktA were used as controls and had no effect on the CL inhibition assay.

Table 9.

Chemiluminescence assay of *in vitro*-activated recombinant leukotoxin and comparison with *in vivo*-activated toxin.

Activation reaction	**%Inhibition of CL response**
Bovine neutrophils	
1: rLktA+rLktC+CAF	78
2: rLktA+rLktC+Hepes	0
3: rLktA+Hepes	0
4: LktC+Hepes	0
5: CAF+Hepes	0
6: Active rLkt(*in vivo*, control)	100
7: Native Lkt (undiluted, control)	95
Rabbit neutrophils	
1: rLktA+rLktC+CAF	25
2: rLktA+rLktC+Hepes	0
3: rLktA+Hepes	0
4: LktC+Hepes	0
5: CAF+Hepes	0
6: Active rLkt(*in vivo*, control)	0
7: Native Lkt (undiluted, control)	0
Human neutrophils*	
1: rLktA+rLktC+CAF	100
2: rLktA+rLktC+Hepes	0
3: rLktA+Hepes	0
4: LktC+Hepes	0
5: CAF+Hepes	0
6: Active rLkt (*in vivo*, control)	50
7: Native Lkt (undiluted, control)	45

*Samples 1-5 were tested at a dilution of 1 in 200 whereas all other samples were tested at 1 in 1000.

4. DISCUSSION

4.1 *PASTEURELLA HAEMOLYTICA* ISOLATES

In the present study, representatives of all 16 recognised serotypes and 4 bovine untypable strains were obtained from both pneumonic and healthy cattle, with the aim of detecting differences and similarities which could prove useful in characterisation of LktA and its role in disease. The initial part of the present study confirmed previous findings that all strains were correctly identified as *P. haemolytica* (Ali *et al.*, 1992; Azad *et al.*, 1992; Davies *et al.*, 1992). For some strains the serotypes were also confirmed by the indirect haemagglutination test.

Many commercial media, such as API 20 NE, are available for identification of *Enterobacteriaceae* and are suitable for preliminary identification of *P. haemolytica*, but for confirmation of this bacterium additional tests are necessary. Production of haemolysis on blood agar and growth on MacConkey's medium differentiated *P. haemolytica* from other pasteurellae and most related organisms. *Actinobacillus* species however, are haemolytic and grow on MacConkey's medium but also produce urease. Indole production was found in the *P. multocida* strain but not in *P. haemolytica* isolates (except Ph6). In identification of *P. haemolytica* by the API 20 NE system, most of the tests were negative. However, typical positive and consistent reactions of *P. haemolytica* were found in the reduction of nitrates to nitrites, production of ß-galactosidase (majority of strains) and oxidase. The above results showed that the strains to be used in the present study were confirmed as *P. haemolytica,* except possibly Ph6, which was atypical in other characteristics but was serotype A1 (Ali *et al.*, 1992) and possessed one plasmid in common with other *P. haemolytica* isolates (Azad *et al.*, 1992).

4.2 PRODUCTION OF NATIVE AND RECOMBINANT LEUKOTOXIN (rLktA)

4.2.1 Cultural conditions affecting production of native leukotoxin

Thirty three *P. haemolytica* isolates, including different serotypes (1-16) and four untypables, were examined for LktA production in culture supernates. The production of LktA was optimised by growing the bacteria for different times in different media and under different condition of aeration, temperature, iron and pH.

Growth media. The production of some bacterial toxins is known to be regulated by the composition of the growth medium and in previous studies *P. haemolytica* has been grown in many different media to produce LktA: brain-heart infusion broth (BHIB) (Strathdee and Lo, 1989), dialysed BHIB (DBHIB) (Chang *et al.*, 1987b); RPMI alone (Kaehler *et al.*, 1980); RPMI containing 0.05, 0.1 and 0.5% bovine serum albumin (Confer and Durham, 1992); RPMI containing 2.5% fetal calf serum (FCS), RPMI containing 700 µg/ml $FeSO_4$, RPMI containing FCS and $FeSO_4$ (Gatewood *et al.*, 1994); growth of the bacteria in BHIB for 4.5 h and then in RPMI 1640 containing 7% FCS (Shewen and Wilkie, 1982). The growth curves of bacteria in RPMI alone and with different concentrations of BSA were similar, but maximum LktA activity was found when bacteria were grown in RPMI containing 0.5% BSA (Confer and Durham, 1992). In different media, such as BHIB, RPMI, RPMI + FCA and RPMI + FCS + $FeSO_4$, LktA activity was greatest in RPMI containing FCS and final optical density of the culture in this medium was greater than in the other media (Gatewood *et al.*, 1994).

In the present study comparing BHIB and DBHIB, more toxin was produced in BHIB. Bacterial growth curves in the various media were similar. However, LktA activity in culture supernates varied between the various

media tested. The yield of LktA was greatest after growth in RPMI + FCS 7% for 2 h, but this was not markedly greater than the yield obtained by growth in BHIB. In view of the technical complexity and expense of the RPMI method, BHIB was chosen for further studies. In addition, the presence of serum protein in RPMI + FCS caused problems in interpretation of gel profiles, caused cross-reactions in immunoblots and also affected CL responses of some species of neutrophils, which did not occur with BHIB as the growth medium.

4.2.1.1 Age of culture

The age of the culture was found to have a major influence on LktA yield from different strains. For example, the time of maximum production of LktA by isolate Ph2 was 6 h whereas for isolate Ph72 it was 8 h, although this represented the end of log phase in both cases. Thus, the amount of LktA activity in these culture supernate samples closely paralleled bacterial growth. There was good agreement between the amount of LktA antigen produced in the culture supernate as judged by SDS-PAGE and immunoblotting and the amount of LktA activity detected by the CL inhibition assay. These results indicate that production of LktA in BHIB was dependent on active growth of bacteria. As the culture aged, there was a marked decline in toxin activity and this might have been due to degradation of the toxin. The same result was reported by Baluyut *et al.* (1981). In another report, the optimum time for maximal toxin production for one isolate of *P. haemolytica* A1 was found to be in the logarithmic phase of bacterial growth, whereas culture supernates from stationary phase contained a minimal amount of active LktA (Chang *et al.*, 1986b). Strathdee and Lo (1989) also reported that secretion of leukotoxin started in early log phase and continued throughout log phase and in stationary phase, secretion of toxin began to decrease (Strathdee and Lo,

1989).

Bacterial genes respond to a number of environmental changes such as temperature shock, osmotic shock and anaerobiosis. Environmental stress induces changes in DNA supercoiling and thus provides a global communication network, coordinating the cell's responses to different stresses. When cells are shifted from one condition to another such as changing from aerobic to anaerobic growth, the rate of synthesis of many proteins is altered. Osmolarity is involved in expression of the staphylococcal enterotoxin type C gene (Regassa and Betley, 1993) but not type A. In *E. coli*, low osmolarity caused a considerable enhancement of haemolysin production (Carmona *et al.*, 1993). In this study, the ability of *P. haemolytica* to response metabolically to changes in aeration, temperature, osmolarity and pH was tested.

4.2.1.2 Aeration conditions

Growth of the *P. haemolytica* Ph2 isolate under various conditions of aeration was monitored by spectrophotometry and LktA production and toxic activity was measured by immunoblotting and CL inhibition assays. The maximum yield of toxin and maximal growth was obtained with high and very high aeration. When LktA activity of these same samples was monitored by CL assay, similar results were obtained. The bacterium produced most LktA activity with very high and high aeration. There are no previous reports on the effect of aeration on toxin production from *P. haemolytica*, although Davies *et al.* (1992) showed differences in OMP and LPS profiles of *P. haemolytica* strains under the same conditions.

4.2.1.3 Temperature

The production of LktA at different temperatures was examined. *P.*

haemolytica produced more toxin and grew optimally when grown at 37°C. At 41 °C production of LktA decreased and no LktA was detected at 20°C. In previous work, Strathdee and Lo (1989) used immunoblotting with monoclonal antibody to show that secretion of LktA by a *P. haemolytica* A1 strain was not different at 37 and 40 °C, but it was slightly decreased at 35 °C and not detected at 30 °C. These slightly different results could be due to strain differences or to the sensivity of the detection system used.

4.2.1.4 Stability of LktA

Temperature also has a profound effect on LktA activity. The toxicity of LktA was destroyed by boiling for 30 min or autoclaving for 15 min. The same result was reported by Sutherland and Redmond (1986). These workers also showed that heating the LktA at 60 °C for 30 min did not affect its cytotoxicity. In the present work, however, the toxicity in culture supernate samples decreased even at -70 °C after 20 days, and they lost more than half of their activity at room temperature for 7.5 h. These different results might be due to the use of a less sensitive method for measuring LktA activity by Sutherland and Redmond (1986). The results reported agree with a previous report by Chang *et al.* (1986b) who showed that the toxicity of LktA decreased by approximately 10-20% per hour at 37 °C and with prolonged incubation at 22 °C for 2 days and at 4 °C for 5 days, the activity of LktA was completely destroyed.

Iron. *P. haemolytica* requires iron for growth and production of LktA. According to Gentry *et al.* (1986) production of LktA required a higher concentration of iron than is needed for growth of *P. haemolytica* . It was shown that secretion of LktA in the presence of 50 μM EDDA in BHIB was reduced whereas secretion of LktA was not affected at 25 μM EDDA (Strathdee and Lo, 1989; Gentry *et al.*, 1986)

To show the effect of iron on production and secretion of LktA, *P. haemolytica* Ph2 was grown in the presence of different concentrations of iron and 2-2'dipyridyl in BHIB. The production of LktA was not influenced by addition of either 1 mM iron or 200 mM 2,2'-dipyridyl to BHIB. Neither significantly affected growth of the organism nor the production of LktA. These results indicate that the amount of iron in BHIB was sufficient for growth of bacteria and production and secretion of LktA. This difference in results from those of Gentry *et al.* (1986) and Strathdee and Lo (1989) might be due to using different media or a different iron chelator or perhaps 2-2'dipyridyl could not remove all the iron from BHIB. However, there was a significant alteration in the expression of three iron-regulated proteins when *P. haemolytica* was grown in BHIB with 2,2'-dipyridyl at the same concentration (Davies *et al.*, 1992).

Osmolarity. Different concentrations of NaCl in BHIB were tested to determine the effect of osmolarity on growth and LktA production. *P. haemolytica* produced LktA normally when grown in 0.1, 0.3 and 0.5 M NaCl. However, the bacteria did not produce LktA in the presence of 0.8, 1 or 1.2 M and the highest concentrations (≥1.0 M) prevented growth of the organism. However, there was only slight retardation of growth of the bacteria in lower salt concentrations. There are no previous reports of effect of high salt on LktA production but such conditions have been reported to reduce HlyA production in *E. coli* (Carmona *et al.*, 1993).

Different concentrations of glucose and sucrose were also investigated. Strain Ph2 produced similar amounts of LktA when it was grown in BHIB with different concentration of the sugars (0-1 M).

pH. Production of LktA at different pH values in BHIB was examined. *P. haemolytica* produced more toxin in alkaline (pH=8) medium than in acidic (pH=6) medium but the amount in the former was comparable to that

produced in normal BHIB (pH 7.3). This result confirms previous results that secretion of LktA was reduced at low pH (<7) (Strathdee and Lo, 1989).

Antibiotics. *In-vivo*, the DNA of all prokaryotes is reported to be in the supercoiled state. The degree of supercoiling may have an effect on expression of certain genes and is maintained by a balance of the actions of DNA gyrase and DNA topoisomerase I. Supercoiling may be affected by environmental conditions and these in turn can thus alter gene expression (Dorman and Ni Bhriain, 1993). DNA gyrase is an essential enzyme in *E. coli* and is inhibited by antibiotics coumermycin A1 and novobiocin. To investigate any effect of these antibiotics on LktA production, *P. haemolytica* Ph2 was grown in BHIB in the presence of novobiocin and coumeromycin A1. The production of LktA was not altered by the presence of different concentration of these antibiotics at concentration which did not inhibit growth (<25 μg/ml for novobiocin and <20 μg/ml for coumeromycin A1).

4.2.1.5 The effect of serial passage of *P. haemolytica* on production of LktA.

In the present study it was found that some strains of *P. haemolytica* produced only low amounts of active toxin in culture supernates. To show whether toxin production or toxic activity decreased after repeated subculture of *P. haemolytica,* strains Ph2 and Ph10 were passaged 40 times on BHIA, a process which took 3 months. Serial passage did not affect the LktA activity and quantity of LktA protein present in culture supernates of the organism during and after passage, as judged by the CL-inhibition assay and immunoblotting, respectively. These results indicate that *P. haemolytica* does not lose its ability to produce active LktA on subculture and freshly-isolated cultures of this organism are not necessary for assessing toxin production. Similar results have been reported by Gentry *et al.* (1987).

Aggregation of LktA. In an attempt to purify the LktA from BHIB culture supernates, different Amicon ultrafiltration membranes were used. Although the mol. wt of leukotoxin is 105 kDa, as judged by SDS-PAGE, it did not pass through an Amicon ultrafiltration membrane with a cut-off of 300 kDa. A similar result was reported by (Baluyut *et al.*, 1981). The mol. wt of LktA was reported to be around 1,000,000 as judged by gel filtration chromatography with Sephacryl S400HR and when 3 mM guanidine was added, the apparent mol. wt of toxin decreased to 800 kDa (Clinkenbeard and Clinkenbeard, 1993). The LktA appears therefore to be combined with some other cell component or in an aggregated state. To try to reverse the aggregation, the effect of various agents were tested. Thus, LktA was treated with 2% SDS (to prevent hydrophobic interactions), 2% zwittergent (zwitterionic detergent), EGTA (1 mM to remove calcium) and 8 M urea (known to prohibit aggregation of *B. pertussis* CyaA and other RTX toxins). They had no effect on the aggregation of LktA as judged by the failure of the treated toxin to pass through a 300 kDa cut-off Amicon ultrafiltration membrane.

4.2.2 Production of recombinant LktA (rLktA) from *E. coli*

4.2.2.1 Preparation of urea extracts

To produce large amounts of leukotoxin, the *lktA* and *lktC* genes were transferred to *E. coli*. These two genes are responsible for production of active toxin and two other genes (*lktB* and *lktD*) are necessary for secretion of leukotoxin. In absence of *lktC*, bacteria produce a non-activated form of LktA. In the absence of *B* and *D* gene products, LktA accumulates inside the cells as inclusion bodies. In this study, *lktA* alone or with *lktC,* was transferred to different *E. coli* strains to produce rLktA. Various methods were used for lysing the cells to extract the rLktA from within. Method 1, involving sodium

deoxycholate, was inconvenient because of the viscosity of this compound. Method 3, involving sonication, is suitable for small volumes of samples. A combination of method 2, involving lysozyme and glycerol, and method 3 gave the best lysis of cells to allow recovery of toxin which was relatively uncontaminated with other cell products.

Monoclonal antibody was used for detection of rLktA and the mol. wt of both active or non-activated (rLktA) was 105 kDa as judged by immunoblotting. *E. coli* strains HMS174 or SY327λ pir containing plasmid pGW42 (encoding active rLktA) produced protein of 105 kDa that reacted with the monoclonal antibody. However, with *E. coli* DH5aF'IQ, a protein >120 kDa was produced in addition to the 105 kDa protein. Both forms were recognised by an LktA-specific monoclonal antibody. The higher molecular weight species is most likely to be a fusion protein of LktA (105 kDa) and LktC (19.8 kDa). Translation of *lktC* is terminated by a UAG (amber) stop codon. Suppression of UAG would produce a protein in which LktC is linked to LktA by a 6 amino acid sequence translated from the inter-cistronic region.

4.3 DEVELOPMENT OF THE CL ASSAY

For evaluation of the effect of LktA on leukocytes, the CL inhibition assay was used. This assay has been reported to be 100 and 2000 times more sensitive than the Cr-release assay and the trypan blue dye exclusion (TBDE) assay, respectively (Chang and Renshaw, 1986). The CL assay is a measure of functional activation of phagocytic cells which can be inhibited by killing the cells. To confirm killing of leukocytes or to determine other functional changes induced by LktA, additional assays such as TBDE, cell tracking and chemotaxis assays were used in this study.

4.3.1 Different preparations of target cells

Preliminary work was aimed at finding suitable preparations of target cells for LktA activity, for assay by CL-inhibition. The most important consideration for each technique was the purity and viability of the resulting cell preparation. Bovine whole blood, isolated lymphocytes, monocytes and neutrophils were used to measure the normal CL response. Small numbers of contaminating erythrocytes in the neutrophil preparation can cause significant light arrest and it is recommended that erythrocytes should be removed as far as possible (Easmon *et al.*, 1980). Hypotonic lysis and isotonic ammonium chloride are often used for the lysis of the RBCs.

The hypotonic lysis method gave rise to several problems. It produced neutrophil suspensions with low viability and purity because of the difficulty in restoring isotonic conditions sufficiently quickly. A method which was more suitable for preparation of neutrophils for CL assay used Histopaque and subsequently ice-cold NH_4Cl medium. This resulted in a preparation containing >95% neutrophils and with >98% viability. However, neutrophils prepared in this way do not respond to interleukin 8 (IL-8) and are not suitable for cell tracking assays (S. Chettibi, personal communication). This may be due to the movement of NH_4 across the plasma membrane. However, extracellular NH_4Cl has no effect on generation of superoxide and the CL response (Activation of NADPH oxidase p130).

For cell tracking assays another method was used involving Percoll and Histopaque. The resulting cell suspensions contained >85% neutrophils with >98% viability. The problem with this method, in addition to the lower purity of neutrophils, was contamination with RBCs which might effect the CL response. Another method, adding 5% w/v dextran to a final concentration of 0.5% to whole human blood, was suitable for processing large volumes of blood but the neutrophils were contaminated with RBCs and this method was

not suitable for bovine blood.

Neutrophils prepared with NH_4Cl gave a good CL response to appropriate stimuli. Purified bovine lymphocytes however, did not produce a CL response when incubated with any stimulus. This result agrees with that of Nelson *et al.* (1976) with human lymphocytes. However. since LktA was shown to bind to lymphocytes and these may therefore interfere with the CLI assay, it was thought necessary to remove them from neutrophil preparations. Bovine monocytes produced approximately 1/8th of the CL response produced by the same number of bovine neutrophils when used as target cells. Nelson *et al.* (1976) had shown previously that human monocytes produced approx. one-third of the CL response produced by human neutrophils.

4.3.2 Factors affecting the CL assay

4.3.2.1 Chemiluminescence

Several factors may affect the CL response of neutrophils, including the presence of RBCs, phenol red and fetal calf serum (Easmon *et al.*, 1980). The temperature also has an affect on CL response and at 20 ° C the peak of CL is less than at 37 °C. Human serum albumin (HSA) at concentrations between 0.01 and 1% totally inhibited the CL response (Brihein *et al.*, 1984). In the present study, these factors were avoided in the CL assay.

4.3.2.2 Effect of different chemiluminogenic probes on CL response.

The chemiluminescent signal is normally amplified by adding compounds such as luminol, DNDH or lucigenin to the assay mixture. Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) is a synthetic compound which emits light when oxidized by either peroxides or oxygen radicals. It interacts with the oxidizing species to produce larger, more measurable amounts of light at a

peak wavelength of approximately 425 nm (LKB Application note 100). Lucigenin is sometimes used as an alternative to luminol but needs the presence of divalent cations to function. Lucigenin has been used in the study of a number of cell types including neutrophils, alveolar macrophage and *Acanthamoeba castellanii* (Dahlgren *et al.*, 1985; Ward *et al.*, 1990; Davies *et al.*, 1991a). The results of Edwards (1987) showed that luminol-dependent chemiluminescence is dependent on oxidase activity as well as degranulation (myeloperoxidase activity) whereas lucigenin monitors oxidase activity independently of the extent of degranulation. DNDH is apparently more sensitive than luminol in enhancing the production of light during neutrophil responses.

In the present work, lucigenin and luminol were compared. The peak CL response with lucigenin was much less than with luminol. This could have been due to impermeability of neutrophils to lucigenin as it is a larger molecule (510 daltons) than luminol (177 daltons) and only measures extracellular events (Dahlgren *et al.*, 1985).

4.3.2.3 Effect of different stimuli

The chemiluminescence response of bovine neutrophils to different stimuli i.e. opsonised zymosan, PMA, fMLP, latex beads, and zymosan was measured. Different stimuli act on different cell surface receptors and via different signalling pathways. fMLP could not stimulate CL responses in bovine neutrophils. The lack of response of bovine and ovine neutrophils to fMLP has been reported previously and may be due to the absence of specific receptors on ruminant neutrophils (Carroll *et al.*, 1982). Brihein *et al.* (1984) showed that when human neutrophils were exposed to fMLP, the cell produced two peaks of CL, the first peak within 2 min and the second peak 10 min after addition of stimulus. These 2 peaks were increased by increasing

the luminol concentration. With addition of HSA, the first peak was reduced, but there was no effect on the second peak which indicates that the first peak is a result of an extracellular reaction whereas the second peak is a result of intracellular reaction. Human neutrophils were used as target cells for LktA in the present work but the response to fMLP was rapid and the first peak was sometimes missed. It was more convenient to use PMA as a stimulus with these cells as it gave only one, large peak.

The response of bovine neutrophils to latex beads was weak and PMA induced a transient and rapid response. Opsonised zymosan, however, induced a slow but high and persistent response. In the presence of calcium the response was faster and greater and so 1 mM calcium was added routinely to the CL assay. Non-opsonized zymosan stimulated a CL response in bovine neutrophils but it was lower than the response to opsonized zymosan. The temperature of the stimulus was found to have an effect on CL responses. Addition of ice-cold buffer or a change in temperature of the neutrophil suspension alone could stimulate a CL response and so such changes were avoided. Addition of OZ warmed to room temperature was found to be suitable for these experiments. Increasing the concentration of stimulus (i.e.. OZ, PMA) produced a greater CL response. In individual assays, different neutrophil preparations gave different levels of response and so the stimulus concentration was adjusted when necessary to give a reasonable peak response.

Using DNDH as chemiluminogenic probe, OZ as stimulus and 1 mM calcium was found to be the best conditions in order for bovine and ovine neutrophils to give a CL response.

4.4 CL-INHIBITION ASSAY

The animal source of neutrophils e.g. ovine or bovine and day-to-day

handling variables contributed to variations in susceptibility of neutrophils to the effect of LktA in each experiment. A similar functional variability of neutrophils from different cows has been reported (Maheswaran *et al.*, 1993). However, the relationship between neutrophils and crude leukotoxin concentration was similar for each experiment and culture supernate of Ph2 was used as internal control.

4.4.1 Assay of LktA in *P. haemolytica* isolates

The present study was designed to investigate whether different strains of *P. haemolytica*, used previously in our laboratory and which showed variation in LPS and OMP profiles (Ali *et al.*, 1992; Ali, 1993), produced different amounts of LktA activity.

Loss of LktA activity in the ageing cultures was accompanied by the appearance of immunoreactive material of lower mol. wt than the usual 105 kDa form, as reported by Chang *et al.* (1987b). Samples taken at the end of log phase, which always contained the greater amount of LktA activity, also lost activity on standing and on prolonged storage. For these reasons, it was important to standardise the time of harvest of culture supernates and to assay toxin activity promptly.

Of the 33 strains tested, most (24) were highly or moderately toxic. All 4 of the biotype T (*P. trehalosi*) strains had low toxicity, as did one serotype A1, two A2 and one untypable strain. Strains representative of serotypes 1-12 of *P. haemolytica* have been shown previously to produce leukotoxin activity although some were reported to have only low toxicity (Shewen and Wilkie, 1983; Chang *et al.*, 1987a; Gentry *et al.*, 1988; Gerbig *et al.*, 1992). Some untypable strains did not appear to produce LktA but these has been isolated from chickens rather than ruminants (Chang *et al.*, 1987a) and so their identity may be in doubt. The low toxicity of a T3 strain has been reported

previously (Winfield and Lo, 1991). In the present panel of strains, 9 A1 and 5 A2 strains were tested and both of these serotypes contained high, moderate and low toxin producers. This inter-strain variation within the serotype groupings parallels the variation in outer-membrane protein and lipopolysaccharide profiles in a similar panel of *P. haemolytica* strains (Ali *et al.*, 1992; Ali, 1993; McCluskey *et al.*, 1994). Only one strain, Ph6, produced no detectable toxic activity. This strain is serotype A1 from a pneumonic calf but is atypical in other respects such as its biochemical, outer-membrane protein and lipopolysaccharide profiles (Ali, 1993) and plasmid profile (Azad *et al.*, 1992).

A surprising finding in the present study was that there was no clear relationship between the amount of LktA protein in the culture supernate, as judged by immunoblotting and ELISA, and leukotoxic activity in the CL inhibition assay. For example, the culture supernates from strains Ph14 and 42 contained the 105 kDa LktA protein in amounts comparable to that from strain Ph2 but had little leukotoxic activity. Such differences in toxicity of the LktA preparations could be due to intrinsic differences in the LktA proteins produced by the different strains or due to strain differences in other *Pasteurella* products such as the LktC activator protein or LPS. It has been suggested that LPS may have a role in the haemolytic activity of *E. coli* haemolysin (Welch, 1994) and mutations in LPS biosynthesis have been shown to be responsible for reduction in haemolysin activity (Stanley *et al.*, 1993). There is inter-strain variation in the LPS profiles of our *P. haemolytica* isolates (Ali *et al.*, 1992) but there was no obvious correlation between LktA activity and LPS type. In addition, neither smooth nor rough LPS from *P. haemolytica* alone caused any significant inhibition of the CL response of bovine neutrophils but a synergistic effect with LktA cannot be excluded.

The results of Breider *et al* (1990) showed that *P. haemolytica* produces

a soluble factor, presumed to be lipopolysaccharide, that was directly toxic to bovine pulmonary endothelial cells *in-vivo*. These workers prevented enthothelial cell damage by removal of LPS from culture supernates with polymyxin B. However, in contrast, Maheswaran *et al.* (1993) with the same technique, reported that LktA, not LPS, was responsible for killing the endothelial cells and in the presence of neutrophils killing was increased.

High concentrations of LktA rapidly abolished CL responses and killed the bovine and ovine neutrophils. The same result has been reported by other researchers (Chang and Renshaw, 1986; Czuprynski and Noel, 1990). Czuprynski *et al.* (1991b) reported that a low concentration of LktA stimulated bovine neutrophils. This result, however, contrasted with their previous work that reported low concentrations of LktA did not have any direct activity on CL response (Czuprynski and Noel, 1990). The reason for this different result has not been found (C. Czuprynski, personal communication). In the present study, repeated attempts were made with different concentrations of LktA including recombinant LktA, but no stimulation of CL was obtained in any assay. Perhaps these discrepancies might be due to different preparations of LktA being used or to different degrees of purification of LktA, or an effect of other *P. haemolytica* components.

4.4.2 Recombinant LktA (rLktA)

The leukotoxin activity of non-active and active forms of rLktA was evaluated by CL inhibition assay. Recombinant LktA was produced in *E. coli* in large amounts as inclusion bodies and was then extracted with urea. The active form of rLktA prepared in this way was considerably more toxic in the CL assay than the native LktA from culture supernates in BHIB. A 1 in 5000 dilution of the active rLktA had approximately the same CL-inhibition activity

as the native LktA from strain Ph2.

Active rLktA inhibited the CL response of the bovine or ovine neutrophils to OZ at a concentration of 1 μg protein/ml. However, the non-activated form of rLktA (rLktA) or a preparation of rLktC used as controls, at the same protein concentration or higher (1-35 μg/ml), stimulated a CL response when mixed with neutrophils at time 0 i.e. before the addition of OZ. The subsequent CL responses to OZ were not affected by either inactive rLktA or rLktC and there was no CL inhibition. The reason for stimulation of bovine neutrophils by inactive rLktA was not due to leukotoxin because the same result was found with rLktC which did not contain any LktA. This stimulation must be due to some other component of *E. coli* but it was not due to urea present in urea extract. Although high concentrations of active rLktA inhibited the CL response, they also prohibited the effect of other components of *E. coli* from stimulating bovine neutrophils. Serial dilution of active rLktA did not cause any stimulation of the response of bovine neutrophils, presumably due to the dilution of these components. This supports the idea that the reported stimulation of neutrophils by LktA may have been due to another component in the preparation.

Active rLktA protein was obtained from *E. coli* containing either the *lktA* and *lktC* genes on one plasmid and under control of the same promoter or on 2 separate plasmids. The activity of rLktA produced by *E. coli* containing one plasmid was greater than that from *E. coli* containing two plasmids. The lower activity of the latter was possibly due to a gene dosage effect because of the need to maintain two separate plasmids instead of one in the cell.

4.4.3 Effect of LktA on different target cell types

Because of the inconvenience of requiring freshly-drawn ruminant blood for preparing neutrophils for CL assay, an attempt was made to find a more

convenient target cell. Although LktA killed the bovine lymphoma cell line (BL3), as judged by trypan blue dye exclusion or release of lactate dehydrogenase as reported previously (Clinkenbeard *et al.,* 1989a), these cells did not produce a respiratory burst in response to any stimulus tested and with various chemiluminogenic probes.

The effects of native and rLktA were investigated with non-ruminant cells, including human, rabbit and guinea pig neutrophils, and a mouse macrophage cell line (J774.2) in CL-inhibition assay. None of the LktA preparations had any effect, either inhibitory or stimulatory, on J774.2 cells, on rabbit or guinea pig neutrophils, even at 10-fold high concentration than those used with bovine neutrophils. A similar lack of effect of LktA has been reported on leukocytes from pigs, horses (O'Brien and Duffus, 1987) and man (Kaehler *et al.,* 1980). Although previous reports have suggested that the leukotoxin is specific for ruminant leukocytes, in the present study native and rLktA caused an inhibition of human neutrophil CL responses. The effect on human and bovine neutrophils, however, was different in that complete CL-inhibition was not obtained, even at highest doses tested, and the human neutrophils were not killed, as judged by trypan blue dye exclusion and as seen in the cell tracking assay. An unexplained finding was that rLktA from *E. coli* strain DH5αF'IQ did not have any effect on human neutrophils, even though it was highly active on bovine neutrophils. This may have been related to the fact that a fusion protein of LktC and LktA was produced by this strains in addition to normal LktA. Non-activated rLktA had no inhibitory or stimulatory effect on human neutrophils. Apart from the finding with rLktA produced by *E. coli* strain DH5αF'IQ, these results indicate that LktA, not other components of *E. coli* or *P. haemolytica,* has some effect on human neutrophils. This will be discussed further in the section of leukocyte morphology and movement.

4.5 ANTI-LKT ANTIBODIES

A number of different antibodies were used in this study to characterize the LktA in terms of its mol. wt, amount and activity. These antibodies included bovine convalescent, rabbit polyclonal antisera raised in the present study, and monoclonals against various RTX toxins.

Previous work had shown that serum from adult cattle and serum from calves immunized with log-phase culture supernates of *P. haemolytica* completely neutralized LktA activity (Baluyut *et al.*, 1981), whereas FCS and neonatal calf serum (before immunization) did not. Similar results had been found with convalescent serum after experimental challenge (Ali, 1993) or from healthy cattle vaccinated with *P. haemolytica* whole cells (Gentry *et al.*, 1988). These results were confirmed by the present investigation in which bovine convalescent serum neutralized the activity of LktA of all *P. haemolytica* culture supernates tested. In addition, the rLktA and culture supernates of *P. haemolytica* A 1 (Ph2 and Ph10) had no effect on bovine neutrophils in the presence of a polyclonal antiserum raised against the 105 kDa rLktA (active) purified by preparative SDS-PAGE. However, this antibody only partially inhibited the toxicity of culture supernates of other strains such as *P. haemolytica* NCTC 10634, and the culture supernate of Ph146 was not inhibited even with increased amounts of antibody. In the latter case, however, the CL response of bovine neutrophils to OZ was affected slightly by antiserum alone. The lack of neutralisation of LktA from Ph146 by this polyclonal antibody was paralleled by its lack of reactivity in ELISA. It has been reported that rabbit sera produced against formalin-killed whole *P. haemolytica* from serotypes 1-12 were capable of neutralizing the homologous toxins (Shewen and Wilkie, 1983). The reason for incomplete neutralization in some cases may have been due to the method of preparation of antisera because whole bacteria contain little toxin. However, they showed

complete neutralization of homologous toxin with rabbit antisera raised against lyophilized toxin. In this case, all sera, except that against one strain, also neutralized heterologous toxin but less effectively than homologous toxin. Gentry *et al.* (1988) found that there was no cross-neutralization of LktA between their different strains of *P. haemolytica*. These results indicate that there are differences in structure in LktA proteins produced by different *P. haemolytica* isolates This is also supported by Burows et al. (1993) who describe seven LktA variants on the basis of southern blot analysis of chromosomal DNA restriction enzyme digests. Another possibility for failure in cross-neutralization in the present work is that the polyclonal sera were raised against denatured recombinant protein which would be structurally different from the native proteins.

Although RTX toxins share some regions of homology, there are some differences. RTX toxins possess a series of glycine-rich amino acid repeats within the toxin structure and it has been reported that the repeat region is necessary for haemolytic activity of HlyA (Ludwig *et al.*, 1988; Boehm *et al.*, 1990b). The number of glycine-rich repeat units varies in different RTX toxins e.g. CyaA has 41 glycine-rich repeats, *P. haemolytica* LktA has 6 repeat units and HlyA has 11 repeat units. There is no information about the glycine-rich repeats in other isolates and whether this domain is the same in all 16 *P. haemolytica* serotypes and untypable strains

Although LktA was detected in immunoblotting by polyclonal antibodies raised against the other RTX toxins such as CyaA, these polyclonals did not prohibit the effect of LktA on target cells. Different monoclonal antibodies, to Lkt, CyaA and Apx toxins, were used to detect native and rLktA and all cross-reacted with Lkt. However, none of them could completely neutralize the effect of LktA on target cells as judged by CL assay. Gerbig *et al.* (1992) reported that of six hybridoma monoclonal antibodies

raised against LktA from serotype A1, only one of them could neutralize leukotoxin produced by serotypes A1, A5, A6, A7, A9 and A12 but this antibody did not neutralize LktA from A2 and the T biotype. The epitope recognized by this monoclonal antibody was localized between amino acids 768-939. The same result was reported by Gentry and Srikumaran (1991) in that of five monoclonal antibodies raised against LktA from serotype A1, only two of them could neutralize the leukotoxin of *P. haemolytica* A1. In the present study the T biotype had low toxic activity but this was not neutralized by the monoclonal antibodies, possibly due to different domains being responsible for leukotoxin activity.

From 12 monoclonal antibodies produced against HlyA, only five of them neutralized haemolytic activity of HlyA to varying degrees. The epitopes for these neutralizing antibodies were found to reside within the following HlyA regions: two monoclonal antibodies recognized amino acids 2-160; one, amino acids 161-194; one, amino acids 518-598; one, amino acids 626-726 (Pellett *et al.*, 1990). These results imply that a number of different domains are responsible for HlyA activity and, because of the functional similarity between the RTX toxins, this would be expected to occur in Lkt.

4.6 VARIATION IN AMOUNT AND MOL. WT OF LKT PRODUCED BY DIFFERENT STRAINS OF *P. HAEMOLYTICA*

Differences in mol. wt and toxicity of the leukotoxin from different serotypes have been reported previously, although in most studies only a single strain of each serotype was examined (Shewen and Wilkie, 1983; Lo, 1988; Gerbig *et al.*, 1992; Burrows *et al.*, 1993). LktA produced by a serotype T3 strain was significantly less toxic for bovine leukocytes than LktA from serotype A1 and had a slightly higher mol. wt (Winfield and Lo, 1991). Recently, Burrows *et al.* (1993) showed by SDS-PAGE and immunoblotting

that reference strains of all 16 serotypes produced LktA proteins that were antigenically related and had similar, but not identical, mol. wt values. Other workers have demonstrated some antigenic differences in the LktA proteins from certain serotypes by antibody neutralisation tests (Shewen and Wilkie, 1983; Gerbig *et al.*, 1992) and genetic differences in the *LktA* determinants were revealed by Southern blotting with the serotype 1 determinant as a probe (Burrows *et al.*, 1993). In this study, antigenic differences in the leukotoxin proteins could account for the apparent discrepancies between the amounts of some of the LktA antigens detected by immunoblotting and by ELISA. With preparations from strains Ph146 and Ph152, for example, bands were clearly visible in the immunoblots and yet little antigen was detected by ELISA. This may relate to the fact that the ELISA system used here depended on the interaction of two antibodies with the toxin, neither of which were homologous, although both antibodies gave similar results in immunoblotting.

In the present study, most strains produced LktA of 105 kDa but four strains, (Ph42, Ph44, 10634 and Ph152) produced a higher mol. wt (108 kDa) form. However, there was no obvious relationship between the LktAs with the higher mol. wt and their toxic activities or the characteristics of the producing strain (table 1). Thus, Ph42 is a bovine A2 isolate from pneumonic lung and its culture supernate had low toxicity; Ph44 is a bovine A2 strain but from the nasopharynx of a healthy animal and the sample had moderate toxicity; Ph54 is an ovine A7 isolate from a pneumonic lung and its culture supernate was highly toxic and a bold LktA band was evident in the immunoblot; Ph152 is an untypable bovine lung isolate and had low toxicity.

Although the mol. wt of *P. haemolytica* LktA is 105 kDa in SDS-PAGE and immunoblotting, additional fainter bands at approximately 95 and 97 kDa were sometimes seen in different preparations of LktA from different isolates. Similar results have been reported by other researchers (Chang *et al.*, 1987b;

Gentry and Srikumaran, 1991) and the bands are presumed to be breakdown products of the 105 kDa LktA.

4.7 SPECIFICITY OF THE CL ASSAY FOR LKT

In present study the specificity of the CL assay for LktA was shown in various ways:

a) culture supernate of *P. multocida* NCTC 10322, which presumably contained cell products common to all *Pasteurella* species, had no effect on the CL response.

b) In order to determine whether the toxicity of culture supernates of *P. haemolytica* for target cells was due to, or affected by, any contaminating LPS, additional studies were done: *P. haemolytica* culture supernate or rLktA boiled for 30 min had no effect on the normal CL response. LPS is known to be heat-stable and would be expected to withstand this treatment.

c) In present study it was shown that *P. haemolytica* released LPS into the culture supernate. To investigate whether the LPS was responsible for CL inhibition in bovine neutrophils, the effect of purified LPS of *P. haemolytica* (rough and smooth) and LPS of *E. coli* (smooth) on bovine neutrophils was investigated. The result of this study found no evidence for linking the purified LPS from *P. haemolytica* or *E. coli* with effects on bovine neutrophils. In other experiments, culture supernate of *P. haemolytica* were treated with polymyxin B to neutralize the activity of LPS. This failed to prevent killing of bovine neutrophils by LktA.

d) Detoxi-gel was also used to remove the LPS from *P. haemolytica* culture supernates. However, the toxicity of culture supernate was decreased, as judged by CL-inhibition assay, after detoxi-gel treatment. Further investigation showed that detoxi-gel removed LktA as well as LPS, as judged by immunoblotting.

e) Further conclusive evidence for the specificity of the CL-inhibition assay for LktA was provided when urea extracts of *E. coli* containing rLktA were tested. Active recombinant toxin killed the bovine neutrophils. However, non-activated rLktA was completely inactive, as were urea extracts containing only LktC and urea extracts of the *E. coli* without recombinant plasmids.

f) The toxicity of culture supernates of *P. haemolytica* Ph2 and from *E. coli* were measured after incubation for 30 min with polyclonal antiserum raised against purified rLktA as judged by the CL-inhibition assay. The antiserum completely inhibited the effect of both preparations of LktA on the neutrophils.

4.8 HAEMOLYTIC ACTIVITIES OF DIFFERENT STRAINS

In the present work, culture supernates and rLktA had low haemolytic activity for bovine and ovine erythrocytes and different strains of *P. haemolytica* had different haemolytic activities. Low haemolytic activity of native and rLktA has been reported previously (Forestier and Welch, 1990; Burrows *et al.*, 1993). However, some workers reported that leukotoxin was not haemolytic for bovine or ovine erythrocytes (Berggren *et al.*, 1981). Thus, the possibilities existed that leukotoxin activity and haemolytic activity of *P. haemolytica* or rLktA may be different. Certainly, the cytocidal activity of LktA is greater and haemolytic activity is weak. The *P. haemolytica* isolate Ph6 which did not produce toxin in culture supernate was non-haemolytic, and the non-activated form of rLktA had neither haemolytic nor leukotoxic activity. The results of this study indicated therefore that LktA has leukotoxic activity as well as haemolytic activity, but different domains of LktA may be responsible for the two activities. These results support the previous suggestion of Forestier and Welch (1990) that haemolytic activity of culture

supernate of *P. haemolytica* is due to LktA. A similar situation occurs with *A. pleuroneumoniae* toxins in that ApxI is both strongly cytotoxic and haemolytic whereas ApxIII is cytotoxic but not haemolytic. However, ApxII is a good cytotoxin but weakly haemolytic (Frey *et al.*, 1993; Tu *et al.*, 1994). Rogel *et al.* (1991) showed that the toxic and haemolytic functions of CyaA were separable and probably mediated by different domains on the molecule.

4.9 CALCIUM AND ACTIVITY OF LKT

Calcium has been shown to have an important role in the activity of RTX toxins but some conflicting results have been reported and there is little information on the role of calcium in LktA activity and cell binding. The calcium can bind to the HlyA and the binding site is the glycine-rich units (residues 739-849) (Boehm *et al.*, 1990a). The conformation of CyaA is changed in the presence of calcium and this change is necessary for its insertion into the target cell membrane and for maximal intoxication of target cells, However, haemolytic activity of CyaA has been shown in the absence of Ca^{2+} and the presence of EGTA (Hewlett *et al.*, 1991). The role of calcium for binding of HlyA to cell membranes was investigated. Ludwig *et al.* (1988) suggested that calcium was necessary for binding of HlyA to natural membranes. Rowe *et al.* (1994) reported that HlyA could bind to BL3 cells under calcium-deficient conditions that would not allow cytolysis and they suggested that calcium may not be necessary for HlyA to initiate an association with leukocyte membranes (Rowe *et al.*, 1994). However, it was previously shown that haemolytic activity of HlyA produced by *E. coli* grown in the absence of calcium was calcium-dependent whereas that produced by *E. coli* grown in the presence of calcium was calcium-independent (Boehm *et al.*, 1990a). The same result was reported by van Leengoed and Dickerson (1992) in that toxin produced by *A.*

pleuropneumoniae in the absence of calcium required calcium both for binding to RBCs and haemolytic activity.

Calcium also binds to LktA and is required for leukotoxin activity at low concentrations of the toxin (Chang *et al.*, 1987b). Cruz *et al.* (1990) suggested that lysis of neutrophils was dependent upon the presence of calcium whereas Clinkenbeard *et al.* (1989a) found that LktA could bind to BL-3 cells in the presence of EGTA. Platelet lysis by *P. haemolytica* A1 culture supernate was found to be Ca^{2+} dependent and that adding increasing amounts of calcium to calcium-free medium increased the toxin activity whereas addition of EGTA to the media decreased toxin activity (Clinkenbeard and Upton, 1991). The release of LDH by LktA from BL3 was blocked in the presence of EGTA but it had no effect on K^+ release or cell swelling (Clinkenbeard *et al.*, 1989a). rLktA had lytic activity for BL3 in the presence of 1 mM calcium, and was inhibited by preincubation with 5 mM EDTA (Cruz *et al.*, 1990).

In present study both *P. haemolytica* and *E. coli,* containing plasmid pGW42, produced LktA as judged by immunoblotting when they were grown in BHIB or 2YT in the presence of EGTA. These organisms also produced LktA when grown in LB medium containing EGTA and no added calcium. The LktA was active when bacteria were grown in BHIB or 2YT but no LktA activity was detected in LB medium. A similar result was reported by van Leengoed and Dickerson (1992) in that secretion of *A. pleuropneumoniae* toxin was independent of calcium but cytolytic activity and binding of this protein to RBCs and neutrophils was dependent on calcium being present either in the growth medium or the assay buffer. These results contrast with finding of Frey and Nicolet (1989) that secretion of 105 kDa haemolysin of *A. pleuropneumoniae* serotype 1 was calcium-dependent.

When the native or recombinant toxin, prepared in the absence of

calcium, was incubated with the bovine or ovine neutrophils in the presence of 1 mM calcium, the toxin became active. This different result with different media was presumably due to the availability of calcium in BHIB even when the EGTA was added. The sub-inhibitory concentration of EGTA, apparently did not remove all of the calcium.

4.10 BINDING OF NATIVE AND RECOMBINANT LKT TO TARGET CELLS.

In this study it was found that the active and non-activated form of rLktA were bound by ruminant cells, including RBCs, mononuclear cells and neutrophils. The same result was found with the non-ruminant cells, human and rabbit blood cells. With the leukocyte membrane preparations, a number of lower mol. wt bands were detected by the anti-rLktA antibody, indicating that LktA was degraded by leukocytes, presumably due to leukocyte proteases. More degradation was found when LktA was incubated with human leukocytes than with bovine presumably because the latter are rapidly killed whereas the former are not killed and in fact are stimulated. In an attempt to inhibit the degradation of LktA by leukocytes, the toxin was incubated in the presence of a cocktail of protease inhibitors. The cocktail did not appear to inhibit toxin degradation.

Active extracellular and intracellular HlyA was reported to bind to RBCs (Oropeza-Wekerle *et al.,* 1989) whereas the non-activated form of HlyA or HlyA in which amino acids 673-726 were deleted, did not bind, suggesting that in the absence of HlyC modification, HlyA does not bind to RBC (Rowe *et al.,* 1994). In contrast, however, it was reported that non-activated CyaA could bind to Jurkat cells as effectively as the active form (Hewlett *et al.,* 1993). In this present study, the same result was found with LktA in that non-activated rLktA bound to erythrocytes as well as to leukocytes, as judged by

immunoblotting.

Although some of the native LktAs have low haemolytic or leukotoxic activities (e.g. these from Ph72, Ph42), they still bound to the target cells (data not shown). In addition, the non-activated form of rLktA bound to RBCs, but it had no detectable haemolytic activity. These results indicate that modification of LktA by LktC was important in lysis of target cells but not for binding to target cells. Another possibility is that the binding domain is different from the domain for lysis of target cells.

The native LktA from *P. haemolytica* Ph2, and rLktA from *E. coli* when grown in BHIB or 2YT media, respectively, was examined for binding to the RBCs at different times and temperatures as well as in the presence of 1 M EGTA. When different temperatures were compared, more toxin was bound to the RBCs at 37 °C and haemolysis occurred. At 4 °C, however, binding but no haemolysis was obtained. A similar result was obtained with mononuclear cells as well as neutrophils. *E. coli* haemolysin can bind to erythrocytes both at 0 and 37 °C (Eberspacher *et al.,* 1989). Similar results have been reported with CyaA in that insertion of toxin into the cell membrane occurred over a wide temperature range (4 °-36 °C) but haemolysis did not occur at the lower temperature (Rogel and Hanski, 1992). These results indicated that the toxin first binds rapidly to the cell surface, with membrane insertion and pore formation following as a second slower step. Thus, the process of binding and pore formation by RTX toxins are two distinct processes that may be temporally-dissociated from each other.

In present study it was found that the LktA prepared in the absence of calcium in LB medium plus EGTA, did not bind to any target cells. Similar results were reported with *A. pleuropneumoniae* toxin by van Leengoed and Dickerson (1992). Thus the work reported here indicated that activation of LktA was not important for cell binding, but that association of Ca^{2+} with the

toxin was necessary. The binding of Ca^{2+} presumably stabilises a domain in the toxin necessary for binding. Recently, Baumann *et al.* (1993) reported the 3-dimensional structure of another protein, alkaline protease from *Pseudomonase aeruginosa*, that contains glycine-rich repeat units. The region forms a β–barrel structure where successive β-turns are stabilised in a spiral by Ca^{2+} ions which bind to the glycine rich repeat units in adjacent stands. A structure such as this is presumably necessary for LktA interaction with target cell membranes.

4.11 EFFECT OF LKT ON LEUKOCYTE MORPHOLOGY AND MOVEMENT

The binding assays showed that leukotoxin binds to various target cells but different effects were found in the CLI assay with ruminant and non-ruminant neutrophils. To confirm the results of CLI assay and binding, the effects of LktA on leukocyte morphology and movement were investigated in a cell tracking assay.

4.11.1 Effect of LktA on bovine leukocytes

None of the LktA preparations (native and rLktA) had any significant effect on the movement of bovine neutrophils. The effect of toxin on bovine neutrophils was seen as cell swelling followed by lysis. In less than 15 min, most the cells were killed by active rLktA. However, during this time, non-activated rLktA, at the same protein concentration, had no effect on bovine leukocytes. Clinkenbeard *et al.* (1989b) had shown previously that native LktA killed BL3 cells by swelling and lysis as shown by measuring the release of LDH. In the present work, the rLktA also killed monocytes and neutrophils but it had no effect on lymphocytes and these were the only cells surviving after 15 min. This result indicates that only lymphocytes are resistant to killing

by LktA. No such effect has been reported previously although LktA is known to prevent lymphocyte proliferation (Majury and Shewen, 1991).

4.11.2 Effect of LktA on human leukocytes

Human leukocytes were treated with active or non-activated rLktA. The surprising finding of the present work was that, although leukotoxin bound to human neutrophils, it did not kill the cells. In fact, the toxin stimulated the migration of these cells. A similar stimulation of movement of neutrophils has been found with fMLP (S. Chettibi, personal communication). With active rLktA, neutrophils appear to move with high speed, and with persistence and a high diffusion coefficient. However, in contrast, with non-activated rLktA, the speed was half that seen with the active form and there was no persistence or diffusion coefficient. This indicates that with active toxin, the cells appear to move in the same direction for sometime before changing direction. This migration could be due to both decreased adhesion to the substrate (an albumin-coated covering) and to increased motility. With non-activated rLktA, the cells were adhering and therefore were not able to displace from the original position.

Inflammatory reactions can be suppressed by glucocorticoids by inhibiting neutrophils accumulation at sites of inflammation (Chettibi *et al.*, 1993). The effect of LktA on migration of human neutrophils might be worth further investigation, as a potential way of intervening in the inflammatory response, because it might induce non-specific movement of neutrophils within the host and prevent the accumulation solely at the site of inflammation. These results with cell movement, where active LktA stimulated movement but did not kill the cells, contrasted with the apparent ability of the toxin to partially inhibit the CL response in human neutrophils. This observation requires more investigation because it suggests that the various

effect of LktA can be dissociated.

4.12 CHEMOTAXIS

In the present study, the chemotactic responses of bovine neutrophils were evaluated by incubation with culture supernates of different isolates of *P. haemolytica*, purified LPS and active and non-activated rLktA. Bovine neutrophils responded chemotactically to all *P. haemolytica* culture supernates but to different extent. There was no direct relationship between CLI activities of culture supernates and chemotactic activity i.e. Ph54 was very toxic but had low chemotactic activity. Similar responses were found to purified LPS of *P. haemolytica* Ph2 as well as active and non-activated rLktA. Others have reported that bovine neutrophils respond chemotactically to culture supernates of *P. haemolytica* A1 and A11 (Mdurvwa and Brunner, 1994). These results indicate that the chemotactic response of bovine neutrophils might be due not only to LktA but also to other components of the bacteria such as LPS.

4.13 *IN-VITRO* ACTIVATION OF RLKT

The first activation *in-vitro* of an RTX toxin was reported for *E. coli* HlyA (Issartel *et al.*, 1991). The process involves fatty acylation and the fatty acid was found to be amide-linked (Stanley *et al.*, 1994). The acylation site on HlyA has been shown to lie between the pore-forming domain and the calcium-binding RTX motif.

In the present studies *in-vitro*, non-activated rLktA from a urea extract of *E. coli* containing plasmid pGW64 was activated by mixing with an LktC preparation from *E. coli* and a cytosolic activating factor (CAF), also an *E. coli* cell extract. The activity of LktC was absolutely required for LktA activation but its exact function is unknown.

LktA activated in this way killed bovine neutrophils and inhibited the CL response but it had some effect on rabbit neutrophils. The LktC and CAF or non-activated LktA were used as controls and did not have any effect on the CL inhibition assay. Recombinant LktC alone cannot activate rLktA, but in the presence of CAF, the non-activated rLktA became active. In the present work the problem with *in-vitro* activation was the quick loss of activity of the activated toxin which after 1 h at 4 °C had lost half of its activity. This might have been due to aggregation of the toxin but further investigation is necessary.

Various acyl groups can activate prohaemolysin but there is no information about which fatty acids are better able to activate LktA. It has been reported that myristoylation succeeded in better activation of CyaA than palmitoylation when proCyaA was activated chemically *in vitro* (Heveker *et al.*, 1994). It was shown that undirected transfer of fatty acid to the CyaA protoxin was able to confer both haemolytic and toxic activities to CyaA. As in the wild-type toxin, calcium was required for activity of the chemically-modified (Heveker *et al.*, 1994).

In this present study, lower LktA activity was found with *in-vitro*-activated toxin than with *in-vivo*-activated rLktA. Similar result have been reported with CyaA modified *in-vitro* and *in-vivo* (Heveker *et al.*, 1994). This suggests that *in-vitro* fatty acylation of the protoxin may involve random modification of residues present in the toxin in contrast to the *in-vivo* modification of specific sites. However, *in vitro* activation deserves further study because it will enable the protoxin to be activated with specific fatty acyl groups. The effect that specific acylation has an biological activity of the toxin can then be tested.

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6. APPENDICES

Appendix 1.

API 20 NE tests, substrates and reactions.

Test	Test code	Test substrate	Reactions involved
1	NO3	potassium nitrate	reduction of nitrates to nitrites
2	TRP	tryptophan	indole production
3	GLU	glucose	acidification
4	ADH	arginine	arginine dihydrolase
5	URE	urea	urease
6	ESC	aesculin	hyrolysis (β-glucosidase)
7	GEL	gelatin	hydrolysis (protease)
8	PNPG	p-nitrophenyl-β-D -glactopyranoside	β-glactosidase
9	GLU	glucose	assimilation
10	ARA	arabinose	assimilation
11	MNE	mannose	assimilation
12	MAN	mannitol	assimilation
13	NAG	N-acetyl-glucosamine	assimilation
14	MAL	maltose	assimilation
15	GNT	gluconate	assimilation
16	CAP	caprate	assimilation
17	ADI	adipate	assimilation
18	MLT	malate	assimilation
19	CIT	citrate	assimilation
20	PAC	phenyl-acetate	assimilation
21	OXI	tetramethyl-p- phenylene diamine	cytochrome oxidase

Appendix 2.

Activity of *P. haemolytica* isolates in API 20 NE

	Activity with API 20 substrates (test number)																				
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Ph2	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Ph6	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Ph30	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Ph42	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
FT4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ph144	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Ph146	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Ph152	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
P. multocid*a* NCTC 10322	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P. multocida Rabbit isolate	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Pseudomonas aeruginosa	-	-	-	+	-	-	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+

Appendix 3.

SODIUM DOOECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

(A) Chemical and reagents.

a. Acrylamide/bisacrylamide

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

b. Lower gel buffer (1.5 M Tris-HCl pH 8.8)

Tris	18.1 g
SDS	0.4 g
Dist. water	100 ml

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

c. Upper gel buffer (0.5 M Tris-HCl pH 6.8)

Tris	6.0 g
SDS	0.4 g
Dist. water	100 ml

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

d. TEMED

e. Ammonium persulphate solution (APS)

A10% solution (100 mg/1 ml dist. water) was made up fresh befor use.

f. Solubilising buffer

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	4 g
Bromophenol blue	0.01 g
Upper buffer (pH 6.8) (c) as 1 in 4 dilution to	100 ml

g. Running buffer (8.3)

Tris	3.03 g
Glycine	14.4 g
SDS	1.0 g
Dist. water	1000 ml

h. Staining solution

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

i. Destaining Solution

Methanol		50 ml
	Glacial acetic acid	75 ml
	Dist. water	875 ml

(B) Slab gel preparations

a. Lower (separating) gel

	Percent acrylamide			
	7.5%	10%	12%	
Acrylamide/Bis (30%)	5	10	15	ml
Lower gel buffer (pH 8.8)	10	10	10	ml
Dis. water	25	20	15	ml
APS (10%)	200	200	200	µl
TEMED	20	20	20	µl

b. Upper (stacking) gel

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

Appendix 4. Buffers and reagents

A. Phosphate-buffered saline (PBS pH 7.38)

0.2 M KH_2PO_4 (Solution A)

KH_2PO_4 (2.7 g) was dissolved in 100 ml of deionised water

0.2 M Na_2HPO_4 (Solution B)

Na_2HPO_4 (2.8 g) was dissolved in 100 ml of deionised water

NaCl	7.99 g
KCl	0.199 g

NaCl (7.99 g) and KCl (0.199 g) were dissolved 930 ml deionised water (Solution C)

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

B. Hanks Hepes

	g/l	final concentration
Sodium cloride	8	140 mM
Potasium chloride	0.4	5 mM
Calcium chloride (6H2O)	0.19	1 mM
Magnesium chloride (6H2O)	0.2	1 mM
D-Glucose	1	5 mM
Hepes	2.3	10 mM

Adjust to pH 7.38 with 5 M NaOH.

C. TEN buffer

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

D. TC buffer

Tris-HCl pH 8.0	50 mM
CaCl2	0.2 mM

E. TSB (Transformation and storage buffer)

TSB is 2 x YT containing;	
PEG	10%
DMSO	5%
$MgCl_2$	10 mM
$MgSO_4$	10 mM

F. Western blotting/Immunoblotting

a. Electroblotting buffer

Tris	3.03 g
Glycine	14.4 g
Methanol 20% (v/v) in dist. water	1000 ml

b. Blot developing substrate solution

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

*DAB is a carcinogen, so handle with care.

c. Ponceau-S solution

Ponceau-S	0.5 g
Glacial acetic acid	1.0 ml
Dist. water	100 ml

G. ELISA BUFFERS AND SOLUTIONS

a. Coating buffer (0.05 M carbonate buffr pH 9.6)

Sodium carbonate	1.59 g
Sodium bicarbonate	2.93 g
Dist. water	1.00 l

b. Washing buffer

PBS	1.00 l
Tween-20	0.05 ml

c. Blocking buffer

PBS	100 ml
Tween-20	0.05 ml
Gelatine	0.50 g

d. Citrate-phosphate buffer

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

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

e. Substrate solution

OPD (O-phenylenediamine, Sigma)

f. Developing solution

Citrate phosphate buffer (pH 5)	50 ml
OPD	17 mg
Hydrogen peroxide	10 µl

g. Reaction stopping solution

12.5% v/v sulphuric acid in dist. water

H. MEDIA

a. 2 x YT

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Dist. water	1 l

Dissolved and autoclaved at 15 lb psi for 15 min.

b. 2 x YT agar

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

Dissolved and autoclaved at 15 lb psi for 15 min.

c. LB media

Tryptone (Oxoid)	10 g
Yeast extract	5 g
NaCl	10 g
H_2O	1 l

Dissolved and autoclaved at 15 lb psi for 15 min.