The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide

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Mutants of *Actinobacillus pleuropneumoniae* strain HK 361 (serotype 2) were isolated which were deficient in type II (Ca^{2+} -dependent) haemolysin activity (Hly^-). Some of the Hly^- mutants secreted a potent, heat-labile extracellular cytotoxic activity against porcine alveolar macrophages. Comparison of cell-free culture supernatant from the parent strain and some Hly^- mutants by SDS-PAGE and immunoblotting revealed the loss of a major extracellular polypeptide of 109 kDa. Two Hly^- mutants which in addition failed to secrete a 120 kDa polypeptide produced no extracellular cytotoxic activity, suggesting that the 120 kDa protein was the cytotoxin. Antiserum raised to the culture supernatant from a Hly^- mutant lacking the 109 kDa polypeptide recognized the 120 kDa band, but not the 109 kDa band, in immunoblots and neutralized the cytotoxic activity, but not the haemolytic activity, of *A. pleuropneumoniae*. The 120 kDa polypeptide and extracellular cytotoxic activity were widespread among *A. pleuropneumoniae* strains, but absent from related bacterial pathogens of the pig: *Actinobacillus suis*, *Haemophilus parasuis* and *Pasteurella multocida*. A clear correlation was found between the presence of the 120 kDa polypeptide and cytotoxic activity in culture supernatants. The cytotoxic activity of all the strains tested was neutralized by antibody to the Hly^- extracellular material and by convalescent pig serum. It is proposed that the 120 kDa polypeptide represents the cytotoxin of *A. pleuropneumoniae*, that it is distinct from the haemolysin, and that it be termed pleurotoxin.

Introduction

Actinobacillus pleuropneumoniae is the aetiological agent of contagious porcine pleuropneumonia (Nicolet, 1986; Sebunya & Saunders, 1983). Evidence for the involvement of one or more toxins in the disease includes the rapid course of acute pleuropneumonia (Shope, 1964; Bertram, 1988), the vascular changes associated with the lesions (Liggett *et al.*, 1987) and the finding that cell-free culture supernatant material can induce lung lesions similar to those of natural infection (Rosendal *et al.*, 1980). The organism is known to secrete both haemolytic and cytotoxic activities which may contribute to the development of lesions, but it is not clear whether these activities are properties of the same protein or are distinct.

Cytotoxic activity of *A. pleuropneumoniae* was first demonstrated *in vitro* by Bendixen *et al.* (1981). Both heat-labile and heat-stable activities were found. Since then, a number of observations of cytotoxic activity have

been reported (Kume *et al.*, 1986; Rosendal *et al.*, 1988; Van Leengoed *et al.*, 1989).

The haemolysin has been the subject of a number of studies which have led to the suggestion that it is a major virulence factor in porcine pleuropneumonia (Martin *et al.*, 1985; Rosendal *et al.*, 1988). The haemolysin of serotype 1 was identified as a protein of approximately 104 kDa (Devenish & Rosendal, 1989; Frey & Nicolet, 1988a). More recently, the gene encoding the haemolysin of serotype 1 has been cloned (Chang *et al.*, 1989; Gygi *et al.*, 1990) and shown to be related to the group of extracellular toxin proteins, including the leucotoxin of *Pasteurella haemolytica*, and the HlyA of *Escherichia coli* (Chang *et al.*, 1987; Strathdee & Lo, 1987). Haemolysins from different serotypes of *A. pleuropneumoniae* differ in their requirements for Ca^{2+} for production, or for activity upon erythrocytes (Frey & Nicolet, 1988b). The activity produced only when Ca^{2+} was present during growth was termed type I haemolysin, while the haemolysin requiring Ca^{2+} for its lytic action (resembling the HlyA activity of *Escherichia coli*; Ludwig *et al.*, 1988; Rennie *et al.*, 1974) was termed type II. However, the haemolysin proteins of all 12 serotypes have since

Abbreviation: PAM, porcine alveolar macrophages.

been shown to be immunologically cross-reactive and of identical size (Devenish *et al.*, 1989), suggesting that they are closely related molecules despite differences in activity.

It was reported by Udeze & Kadis (1988) that the haemolysin of the serotype 1 type strain of *A. pleuropneumoniae*, purified by gel filtration and ion-exchange chromatography, was cytotoxic for a variety of pig leucocytes including alveolar macrophages. The haemolysin was considered to be the primary candidate for the toxic activity against lung macrophages and polymorphonuclear leucocytes (Nicolet, 1990; Chang *et al.*, 1989; Devenish & Rosendal, 1989; Kamp & Van Leengoed, 1989; Udeze *et al.*, 1987). However, some observations suggested that the haemolytic and cytotoxic activities of *A. pleuropneumoniae* varied independently (Kamp & Van Leengoed, 1989; Rosendal *et al.*, 1988) and displayed different properties such as heat stability (Fedorka-Cray *et al.*, 1990). One explanation for this was differences in the biochemical structure of the haemolysin/cytotoxin molecule between strains (Devenish *et al.*, 1989). In the present study we show that cytotoxic activity in a serotype 2 strain is independent of haemolytic activity and identify the polypeptide associated with the cytotoxic activity.

Methods

Bacteria and culture conditions. The bacterial strains used are listed in Table 2. *Actinobacillus pleuropneumoniae* strains were stored in 10% (v/v) skimmed milk at -70°C . Routine culture was made on heated blood agar (prepared with 7%, v/v, horse blood) and liquid cultures were grown in Tryptone Soya Broth (Oxoid) supplemented with $2\text{ }\mu\text{g NAD ml}^{-1}$ (TSB/NAD). *Actinobacillus suis*, *Pasteurella multocida* and *Haemophilus parasuis* were stored lyophilized and cultured in the same media. Cultures were grown initially to the exponential phase (2–3 h). Subcultures were then made (1 in 50) to fresh TSB/NAD and grown with gentle aeration to achieve maximum yields of activity (2–3 h for haemolytic activity, 4–6 h for extracellular cytotoxic activity). The comparative growth rate of cultures was determined by measuring the OD_{550} of the culture at intervals during the growth phase.

CAMP factor. Production of CAMP factor cohaemolysin was determined by cross-streaking with a β -toxicogenic strain of *Staphylococcus aureus* (Frey *et al.*, 1989).

Polyclonal antibodies. Antibody to whole, cell-free culture supernatant was raised in New Zealand White rabbits by intramuscular injection of filtered supernatant (Millex GV, $0.22\text{ }\mu\text{m}$, low protein binding; Millipore) with Freund's incomplete adjuvant. This was followed by three biweekly intravenous injections of the same material without adjuvant (Harlow & Lane, 1988). Animals were bled after a further 7 d. Convalescent pig serum was obtained from a pig recovering from pleuropneumonia in an outbreak with *A. pleuropneumoniae* serotype 3 (strain 6664).

SDS-PAGE and immunoblotting. SDS-PAGE was carried out as previously described (Rycroft & Taylor, 1987). The separating gel consisted of 10% (w/v) acrylamide (acrylamide/bisacrylamide ratio,

75:1) in 0.37 M-Tris/HCl (pH 8.7), 0.1% (w/v) SDS. The stacking gel consisted of 5% (w/v) acrylamide (acrylamide/bisacrylamide ratio, 36:1) in 125 mM-Tris/HCl (pH 6.9), 0.1% (w/v) SDS. Culture supernatant for analysis was concentrated 20-fold by precipitation with ethanol (23%, v/v) for 2 h at 0°C followed by centrifugation for 5 min in a microfuge (11600 g). Supernatant was discarded and pellets were redissolved in 150 mM-NaCl. Samples were adjusted to 60 mM-Tris/HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β -mercaptoethanol and electrophoresed at 25 mA in 25 mM-Tris, 192 mM-glycine (pH 8.3). Proteins were visualized by fixing and staining with 0.25% (w/v) Coomassie blue R in 10% (v/v) acetic acid, 40% (v/v) methanol followed by destaining in the same solvent.

For immunoblotting, culture supernatant was used unconcentrated. Gels were washed in 25 mM-Tris, 192 mM-glycine for 20 min at room temperature. Proteins were electrophoretically transferred onto nitrocellulose in a Trans Blot apparatus (Bio-Rad) in 25 mM-Tris, 192 mM-glycine without methanol. Antigens were probed overnight with whole serum diluted (1 in 200) in 10 mM-Tris/HCl, 150 mM-NaCl, 0.05% (v/v) Tween 20 (TNT) containing 5% skimmed milk. Antigens were detected using horseradish peroxidase conjugated second antibody (goat anti-rabbit or rabbit anti-pig, 1:3000) followed by reaction with substrate [0.05% (w/v) 4-chloro-1-naphthol, 4.4 mM- H_2O_2 in 16% (v/v) methanol, 10 mM-Tris/HCl (pH 8.0), 150 mM-NaCl].

Mutagenesis and screening. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was carried out essentially as described by Miller (1972) except that 100 mM-Tris/maleate buffer (pH 5.8) was used during the period of exposure to NTG. Briefly, exponential-phase bacteria were washed in Tris/maleate buffer and NTG was added to a final concentration of $50\text{ }\mu\text{g ml}^{-1}$. Bacteria were incubated at 37°C for 20 min and diluted in 0.1 M-phosphate-buffered saline (pH 7.2). Mutagenized cultures were spread onto double-layer screening plates comprising a lower layer of Nutrient Agar (Oxoid) containing approximately 5×10^4 c.f.u. ml^{-1} of *Staphylococcus epidermidis* as feeder strain and an upper layer of 5% (v/v) sheep blood agar. Cultures were incubated for 18 h before examination for haemolysin-deficient colonies.

Haemolysin determination and neutralization. Culture supernatant from parent and mutant strains was cleared by centrifugation (10000 g for 10 min at 4°C) and filtered through a Millex-GV filter. Fifty microlitres of culture supernatant was diluted in $350\text{ }\mu\text{l}$ 50 mM-Tris/HCl, 150 mM-NaCl, 23 mM- CaCl_2 (pH 7.2) in a microfuge tube. Then $400\text{ }\mu\text{l}$ of 5% (v/v) sheep erythrocytes, washed twice in 150 mM-NaCl, was added and incubated for 60 min at 37°C . Tubes were mixed gently, centrifuged for 15 s in a microfuge and the A_{541} of the supernatant was measured. Haemolysin concentration showed a linear relationship with A_{541} .

The presence of intracellular haemolysin was examined following ultrasonic lysis of bacteria. Bacteria were centrifuged and resuspended in 0.1 volumes of 50 mM-Tris/HCl, 150 mM-NaCl at 0°C . Bacteria were disrupted by sonication for 30 s; cell breakage was judged complete by the microscopic appearance of the cell suspension. Insoluble material was pelleted in a microfuge and the supernatant was assayed for haemolytic activity as described above. Whole bacteria were analysed for immunologically cross-reactive material by dissolving approximately 5×10^8 actively growing bacteria in 60 mM-Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol at 100°C for 5 min (Gulig *et al.*, 1983). Dissolved proteins were analysed by SDS-PAGE followed by immunoblotting.

Haemolysin-neutralizing activity of serum was determined by mixing $10\text{ }\mu\text{l}$ of test serum or diluted test serum with $90\text{ }\mu\text{l}$ of haemolytically active cell-free culture supernatant. Samples were held at 0°C for 60 min and assayed for haemolytic activity alongside controls treated identically with normal pooled or preimmune serum.

The neutralizing titre was expressed as the reciprocal of the dilution of serum required to achieve at least 50% reduction in haemolytic activity of a standard fresh haemolysin preparation (that giving an A_{541} of approximately 1.3).

Porcine alveolar macrophages (PAM), cytotoxicity determination and neutralization. Alveolar macrophages were obtained by lung lavage of freshly killed 10–16-week-old pigs, clinically and bacteriologically free from respiratory infection. The respiratory tract was removed and approximately 1000 ml of Hanks' Balanced Salts Solution (HBSS; Gibco BRL) was poured into the lungs. A volume of approximately 600 ml was recovered by draining and was centrifuged at 110 *g* for 5 min (Leijh *et al.*, 1986). The recovered cells were resuspended to 10^6 cells ml^{-1} in Minimal Essential Medium (Gibco BRL) with 15% (v/v) newborn calf serum (MEM) and 60 μg gentamicin ml^{-1} . Cells were cultured in 24-well culture plates (Nunc), 10^6 cells per well, in an atmosphere of 3% (v/v) CO_2 in air. The medium was changed after 2 h incubation and the proportion of viable cells, determined by exclusion of Trypan blue dye (see below), was shown to be greater than 99%. PAM were used within 6 h.

Cytotoxic activity was determined by treating freshly prepared PAM in 900 μl MEM with 100 μl of diluted, cell-free culture supernatant and incubating at 37 °C for 60 min. Cells damaged by the cytotoxic activity showed loss of refractility and became detached from the plastic. After incubation the medium was replaced with 0.1% Trypan blue in MEM. The proportion of cells stained by Trypan blue was determined after 30 min. One unit of cytotoxic activity was defined as that quantity of toxin which induced 50% of the cells to stain with Trypan blue or become detached, in a volume of 1.0 ml, after incubation for 60 min at 37 °C. Heat lability of toxic activity was determined by preheating for 5 min before testing. Intracellular cytotoxic activity was examined following ultrasonic lysis of bacteria as described for haemolysin.

Cytotoxic-neutralizing antibody was determined by mixing 50 μl of cytotoxin preparation (80 units ml^{-1}) with an equal volume of serum (or serum diluted in 150 mM-NaCl) at 0 °C for 60 min. The sample was then added to 0.9 ml MEM in a well of PAM as previously described. The neutralization titre was expressed as the reciprocal dilution of serum required to entirely prevent the cytotoxic effect of the test mixture.

Results

Isolation of haemolysin-deficient (Hly⁻) mutants of A. pleuropneumoniae HK361

A. pleuropneumoniae HK 361 (serotype 2) produced type II (Ca^{2+} -dependent) haemolysin activity and cytotoxic activity against porcine alveolar macrophages (PAM). Analysis of the cell-free culture material by SDS-PAGE revealed several polypeptides, including two dominant bands of 109 and 120 kDa (Fig. 1, lane 2). In order to establish the identity of the haemolytic protein we sought Hly⁻ mutants of this strain. Approximately 40000 potential mutants were screened following NTG mutagenesis. Twenty-seven colonies having no zone of haemolysis on screening plates were purified and tested for extracellular haemolysin activity, growth rate, CAMP factor and extracellular polypeptides by SDS-PAGE and immunoblotting.

Expression of extracellular cytotoxin against PAM in Hly⁻ mutants

Eight of the mutants secreted no detectable haemolytic activity: 2.3 h culture fluid showed an A_{541} of <0.01 in the haemolysin assay compared to the value of 1.3 for that from the parent strain, and no haemolytic activity could be detected throughout the growth cycle to 18 h after inoculation. The remaining mutants produced varying low levels of Ca^{2+} -dependent haemolysin activity and were not used further. All eight of the Hly⁻ mutants were CAMP factor positive and showed a

Fig. 1. SDS-PAGE and immunoelectrophoretic blot analysis of cell-free culture fluid of *A. pleuropneumoniae* HK 361 and its Hly⁻ mutants. (a) Coomassie blue staining of extracellular supernatants concentrated 20-fold by ethanol precipitation as described in Methods. Lanes: 1, Molecular mass markers; 2, HK 361; 3–10, Hly⁻ mutants of HK 361. (b) Immunoblot analysis of extracellular supernatants probed with rabbit antibody to culture supernatant of HK 361. Lanes: 1, HK 361; 2, HK 361 Hly⁻ mutant e; 3, HK 361 Hly⁻, cytotoxin-deficient mutant h.

Fig. 2. Effect of cell-free culture supernatants from *A. pleuropneumoniae* HK 361 and mutant strains on fresh porcine alveolar macrophages in culture. Cells were treated with: (a) uninoculated culture fluid, (b) cell-free culture supernatant from *A. pleuropneumoniae* HK 361, (c) cell-free culture supernatant from *A. pleuropneumoniae* HK 361 Hly⁻ mutant e, or (d) cell-free culture supernatant from *A. pleuropneumoniae* HK 361 Hly⁻ mutant h. Note PAM in (b) and (c) detached from the culture dish and lysed; remaining cells were lymphocytes and erythrocytes. Bars 0.1 μm .

growth rate indistinguishable from the parent strain. Four of the eight mutants (e, h, o and t) produced no 109 kDa extracellular polypeptide band in SDS-PAGE or in immunoblots probed with antibody to cell-free culture supernatant of *A. pleuropneumoniae* HK 361 (Fig. 1). Cell-free culture supernatant from the eight Hly⁻ mutants was tested for cytotoxicity against fresh PAM. Six of the mutants (e, i, o, s, t and x) showed potent cytotoxic activity as determined by altered cell morphology and uptake of Trypan Blue dye (Fig. 2). This cytotoxicity was of similar potency to that produced by the parental strain (80 units ml^{-1}).

Loss of cytotoxin in mutants lacking the 120 kDa polypeptide

In addition to loss of the 109 kDa polypeptide, one Hly⁻ mutant (h) also failed to produce the 120 kDa polypeptide. Another (g) produced a small quantity of both 109 and 120 kDa polypeptides (Fig. 1a). Neither mutant produced any detectable extracellular cytotoxic activity (<10 units ml^{-1}). Furthermore, no immunologically cross-reactive 120 kDa band, or cytotoxic activity, could be detected in the cell contents of these mutants.

Characteristics and distribution of A. pleuropneumoniae cytotoxin

The properties of the cytotoxin were studied in the Hly⁻ mutants, where possible interference from haemolytic activity could be excluded. Cytotoxic activity was stable upon heating to 60 °C for 5 min but was completely inactivated at 100 °C. Marked morphological changes occurred in the PAM within 10 min of exposure to the cytotoxin. Cell damage was irreversible after 8 min of exposure to 8 units of toxin; removal of the toxin up to this time prevented the characteristic damage to the cells. Cytotoxicity was neutralized by homologous antiserum raised to culture supernatant of a Hly⁻ mutant (e) (which possessed no haemolysin-neutralizing activity: Table 1) and also by convalescent pig serum from an animal recovering from natural pleuropneumonia due to infection with a serotype 3 strain (Table 1).

Cytotoxic activity was demonstrated in the culture supernatants of 9 out of 11 *A. pleuropneumoniae* strains and serotypes tested including representatives of serotypes 2, 3, 4 and 5 (Table 2). Other serotypes were not available for testing. No cytotoxic activity was detected in isolates of *A. suis*, *Haemophilus parasuis*, and *Pasteur-*

Fig. 3. Comparison of extracellular polypeptides of *A. pleuropneumoniae* isolates representing serotypes 2, 3, 4, and 5, and related porcine bacteria by immunoblot analysis. Cell-free culture fluid was separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with rabbit antibody to culture supernatant of HK 361. Culture fluid from: HK 361 (1); HK 358 (2); HK 353 (3); 6664 (4); DT45 (5); 3245 (6); S1181/70 (7); 9792/3 (8); M62 (9); K17 (10); 266-1920-HAE (11); *Haemophilus parasuis* (12); *A. suis* (13); and *Pasteurella multocida* (14 and 15).

Table 1. Neutralization of haemolytic and cytotoxic activity

Serum	Anti-haemolytic activity*	Anti-cytotoxic activity*
Rabbit (preimmunization)	<10	<2
Rabbit anti-HK 361 cell-free culture supernatant	320	64
Rabbit anti-HK 361e cell-free culture supernatant	<10	64
Convalescent pig†	80	128

* Reciprocal dilution of serum required for neutralization as described in Methods.

† Pig serum from animal recovering from natural pleuropneumonia (infection with strain 6664, serotype 3).

ella multocida of porcine origin. Extracellular cytotoxicity was restricted to those strains of *A. pleuropneumoniae* showing a detectable 120 kDa polypeptide by immunoblot analysis (Fig. 3). Cytotoxic activity from all strains tested was neutralized by antibody to Hly⁻ mutant e.

Similarly, it was neutralized by convalescent pig serum from serotype 3 infection (Table 1).

Discussion

Loss of the 109 kDa polypeptide band in Hly⁻ mutants of *A. pleuropneumoniae* HK 361 indicated that this polypeptide represents the haemolysin molecule or a component of it. The molecular size of this polypeptide agrees approximately with previous estimates of the size of the haemolysin of *A. pleuropneumoniae* serotype 1 as 104 or 105 kDa (Frey & Nicolet, 1988a; Devenish & Rosendal, 1989). However, our estimation of the size is closer to that made by Fedorka-Cray *et al.* (1990) and Chang *et al.* (1989), who reported it to be 110 kDa. Hly⁻ mutants continued to produce CAMP factor cohaemolysin, confirming the report of Frey *et al.* (1989) that the latter is not the haemolysin of *A. pleuropneumoniae*.

It has been suggested that the cytotoxic activity of serotype 1 strains of *A. pleuropneumoniae* may be due to

Table 2. Cytotoxic activity of *A. pleuropneumoniae* and related porcine bacteria

Strain	Cytotoxic activity (units)*	Serotype	Source (reference)
<i>Actinobacillus pleuropneumoniae</i>			
HK 361	>40	2	NCTC† M. Kilian (Kilian <i>et al.</i> , 1978)
HK 353	>80	2	
HK 358	>40	2	
6664	>80	3	
DT45	>80	3	This laboratory (Rycroft & Taylor, 1987)
3245	>80	3	
S1181/70	<10	3	M. Kilian
9792/3	>40	3	This laboratory
M62	>40	4	NCTC
K17	<10	5	T. Inzana (Kilian <i>et al.</i> , 1978)
266-1920-HAE	>10	5	M. Kilian
<i>Actinobacillus suis</i>	<10	—	This laboratory
<i>Haemophilus parasuis</i>	<10	—	
<i>Pasteurella multocida</i>	—	—	
462 (Porcine lung)	<10	—	
463 (Porcine lung)	<10	—	

* Cytotoxic activity was determined on three separate occasions, using cell-free supernatant of 4 h cultures. One unit of cytotoxic activity was defined as the amount required to cause at least 50% of PAM to become detached or to stain with Trypan blue dye in 1.0 ml after 60 min incubation at 37 °C. <10 units ml⁻¹ is the detection limit for the assay.

† National Collection of Type Cultures, Public Health Laboratory Service, Colindale, London, UK.

the haemolysin, as the activities share characteristics such as heat-lability, and sensitivity to trypsin, oxygen and cholesterol (Rosendal *et al.*, 1988). In these experiments, Hly⁻ mutants of strain HK 361 retained the capacity to secrete a potent cytotoxin active against fresh PAM *in vitro*. This is the first clear evidence that the secreted cytotoxin is distinct from the haemolysin produced by this organism. The cytotoxic activity showed similar properties to the activity described by Van Leengoed *et al.* (1989) in being extracellular, heat-labile and active against PAM. Changes in cell morphology at the ultrastructural level were observed after 1 h exposure by these authors. We found gross morphological changes within 10 min at dilutions of 1 in 20 or greater, perhaps reflecting quantitative differences in the potency of the prepared toxin rather than any difference in mode of activity.

The finding of two distinct cell-damaging activities in *A. pleuropneumoniae* does not conflict with the results of others (Rosendal *et al.*, 1988; Kamp & Van Leengoed, 1989) who found cytotoxic and haemolytic activities to be independently variable in several isolates. It must be concluded that in addition to haemolysin, a second potential virulence determinant is produced by this

pathogen. Clearly, if produced *in vivo*, the rapid action of the toxin is likely to confer properties upon the organism which allow it to overcome the cellular defences in the porcine lung and induce the changes associated with the early stages of the infection (Liggett *et al.*, 1987).

A role for lipopolysaccharide (endotoxin) in the development of lesions in porcine pleuropneumonia has been postulated (Sebunya & Saunders, 1983; Udeze *et al.*, 1987). In addition, endotoxin is known to cause cytotoxic effects on alveolar macrophages *in vitro* (Davies & Barsoum, 1980). Lipopolysaccharide was not involved in the cytotoxic activity reported here since the toxicity was lost in the mutants g and h. Furthermore, the toxicity was destroyed by heat. This was in general agreement with Udeze *et al.* (1987), who concluded that a heat-labile factor was involved in lesion development and necrosis.

Our results do not exclude the possibility that type I haemolysin (Frey & Nicolet, 1988b) or haemolysin from other strains, may itself have cytotoxic activity. However, some strains of *A. pleuropneumoniae* (e.g. K17, serotype 5 and S1181/70, serotype 3) produced haemolysin and secreted the 109 kDa haemolysin polypeptide alone. These were not cytotoxic, indicating that in these

strains at least, the haemolysin is not cytotoxic. In addition, the cytotoxic activity secreted from Hly⁻ mutants was identical in potency and effect to that from the parent strain. No additive cytotoxic effect could therefore be attributed to the haemolysin. The relationship of cytotoxin and haemolysin in other serotypes, particularly serotype 1 strains, remains to be resolved, but in view of the very close size and immunological similarity of haemolysins of all the serotypes (Devenish *et al.*, 1989), it is possible that the cytotoxic activity will prove to be distinct in these organisms also.

Three of the Hly⁻ mutants (i, s, and x) continued to secrete both 109 and 120 kDa polypeptides. It is likely that these produce a protein with altered structure which is no longer haemolytically active, perhaps due to point mutation. Such a finding is not unexpected. The haemolysin is highly labile (Frey & Nicolet, 1988*a*), being rapidly denatured without proteolytic cleavage (unpublished observations).

One Hly⁻ mutant (h) lacked both the 109 kDa and 120 kDa bands while another (g) produced very much reduced amounts of these. Both mutants failed to produce any detectable cytotoxic activity while all those retaining the 120 kDa band were cytotoxic. This indicates that the 120 kDa polypeptide represents the cytotoxin of *A. pleuropneumoniae*. In support of this was the correlation between the presence of a 120 kDa band in extracellular culture supernatant from other strains of *A. pleuropneumoniae*, and cytotoxic activity. Further evidence for this was the finding that antibody to the culture supernatant from the Hly⁻ mutant e, lacking the 109 kDa polypeptide, neutralized the cytotoxic activity but did not recognize the 109 kDa band, or neutralize the haemolysin. As the cytotoxic activity and its associated 120 kDa polypeptide were functionally and immunologically distinct from the haemolysin, we suggest the term pleurotoxin for this cytotoxic activity.

Mutants of *A. pleuropneumoniae* lacking cytotoxic activity (and the 120 kDa band) were not directly selected, but present among mutants selected for haemolysin deficiency. Mutation of export function could not account for the simultaneous loss of both phenotypes because no intracellular, cross-reactive polypeptide could be detected in those strains by immunoblotting. More likely, it was the result of linkage between the two genes. It is possible that a polar mutation was responsible for the loss of the non-selected phenotype along with the haemolysin protein. Alternatively, inactivation of a regulatory locus such as *vir* of *Bordetella pertussis* (Weiss & Falkow, 1984), could account for this.

We have found the 120 kDa band to be widely distributed among *A. pleuropneumoniae*. However, it was absent from some strains and from other related bacterial respiratory pathogens of the pig. If this

substance were important in the pathogenicity of porcine pleuropneumonia, one would expect it to be present in all isolates of the organism. However, it was noteworthy that the two strains lacking the toxin were originally isolated from non-respiratory sites and were not responsible for pleuropneumonia in the host animals (Kilian *et al.*, 1978). Surprisingly, the 120 kDa band has not been noted previously as a major extracellular product of *A. pleuropneumoniae*. Devenish *et al.* (1989) did not detect this polypeptide by immunoblotting with convalescent serum, in the supernatants from any of the 10 serotypes. It is possible that the medium used for haemolysin production (RPMI) was not suitable for pleurotoxin production. We have not examined this possibility.

Little information regarding similar extracellular cytotoxic activity in related Gram-negative bacteria is available. Two high-molecular-mass extracellular polypeptides of similar size (100 and 116 kDa) were described by Gulig *et al.* (1984) in *Haemophilus influenzae*. No activities were ascribed to these but they were considered to be strongly immunogenic, a notable feature of the 109 and 120 kDa proteins of *A. pleuropneumoniae*.

Although the haemolysin has been proposed as a major factor responsible for the lesions of pleuropneumonia, the secretion of a powerful toxin capable of rapidly destroying the primary host defence of the porcine lung must constitute an important facet of the invasive behaviour of this organism.

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