BACTERIAL PATHOGENICITY

Characterisation of the leukotoxin produced by different strains of *Pasteurella haemolytica*

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Pasteurella haemolytica isolates from cattle and sheep, including representatives of different serotypes and untypable strains, were examined for leukotoxin (Lkt) production at the end of the log phase of growth in brain heart infusion broth. 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 Lkt protein produced by different strains, as judged by SDS-PAGE, immunoblotting and ELISA. Some strains produced normal amounts of Lkt protein which had only low leukotoxic activity. Most strains produced Lkt 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.

Introduction

Pasteurella haemolytica is responsible for economically important diseases of ruminants including pneumonic pasteurellosis of cattle and sheep [1, 2]. The species comprises two biotypes, A and T, although it has been proposed that biotype T be renamed *P. trehalosi* [3]. *P. haemolytica* of various serotypes and in low numbers is part of the normal flora of the upper respiratory tract of healthy animals. Under conditions of stress due to management and environmental factors or other infectious agents, where lung clearance mechanisms are impaired, the lungs become colonised by the organism and disease ensues. Whereas A1 is the predominant serotype isolated from cattle with pneumonic pasteurellosis, A2 predominates in cases in sheep. Biotype T (*P. trehalosi*) strains cause a pathologically distinct systemic infection of young adult sheep. Approximately 10% of all isolates from cattle and sheep are untypable by the usual method of indirect haemagglutination.

Current vaccines against pneumonic pasteurellosis, including live or killed bacteria or various cell components, do not seem to provide consistent protection. *P. haemolytica* produces various virulence

components that are potential protective antigens. These include leukotoxin (Lkt), lipopolysaccharide (LPS), capsular polysaccharides, fimbriae and various outer-membrane proteins (OMP) [4]. Lkt in particular has attracted much interest as a vaccine component as it is thought to play a central role in the disease process. A naturally occurring antibody response to Lkt has been correlated to disease resistance in the field, and vaccines containing Lkt, along with other components, give some protection against experimental challenge. However, an immune response to Lkt alone does not appear to be sufficient to protect animals from disease [5].

Lkt is one of a group of high mol. wt, calcium-dependent, pore-forming protein toxins produced by several gram-negative bacteria. These toxins show structural, antigenic and genetic homology with the widely studied haemolysin of *Escherichia coli*. They have been designated the RTX (repeats in toxin) family due to the presence of characteristic glycine-rich repeat units at their C-termini [6]. They also have similar mechanisms for their activation and secretion from the bacterial cell. For example, active Lkt is produced as an inactive precursor, the product of the *lktA* gene, and is then activated post-translationally by the *lktC* gene product. The apparent mol. wt of Lkt, as estimated from SDS-PAGE and immunoblotting, is c. 105 kDa [7], which is similar to the 102 kDa predicted from the nucleotide sequence of the *lktA* gene [8].

The RTX toxins vary in their target cell specificity. Some are cytotoxic for a wide variety of eukaryotic cell types whilst others are more specific. Lkt has been reported to act predominantly on leucocytes of ruminant species [9], which thus parallels the susceptibility of ruminants, and the low susceptibility of non-ruminants, to *P. haemolytica* infection. Both lytic and non-lytic effects have been reported. These various effects on leucocytes, resulting in impairment of host defences and release of lysosomal contents and toxic oxygen radicals, are thought to be important in the harmful inflammatory reactions and lung damage of pneumonic pasteurellosis [10]. One of the effects of Lkt on neutrophils *in vitro* is the suppression of their respiratory burst in response to stimuli such as opsonised zymosan. This can be measured as inhibition of a chemiluminescence response and provides a sensitive and convenient assay for the toxin [11, 12].

The aim of the present investigation was to further an understanding of the role of Lkt in the pathogenesis and immunity of pneumonic pasteurellosis. In recent work from this laboratory, analysis of outer-membrane

protein and lipopolysaccharide profiles of a large number of *P. haemolytica* isolates by electrophoresis and immunoblotting has shown that there is considerable inter-strain variation within the existing serotype groupings [13–15]. In the present study, a similar number of *P. haemolytica* isolates, including representatives of all serotypes and untypable strains, was examined for Lkt production.

Materials and methods

Bacteria

Many of the strains have been used in previous studies in this laboratory [13, 16–18] and a brief description of some of their important characteristics is given in Table 1. Strains prefixed with Ph (except 142, 252 and 706) were collected from various parts of Scotland, from pneumonic and healthy cattle, over the period 1982–85. Strains with five-number designations (10632, etc.), were NCTC strains and all were ovine isolates. The remainder were a mixture of ovine and bovine isolates from the collection of Dr W. Donachie, Moredun Research Institute, Edinburgh.

Table 1. Characteristics of strains used in this study

Laboratory designation	Serotype	Species of origin	Isolation	Disease status of host	Age of culture (h)*	Leukotoxic activity: percentage inhibition of CL response of neutrophils† (SD)	
						Bovine	Ovine
Ph 2	A1	Bovine	LRT	Pneumonic	6.0	97 (0.2)	97 (0.5)
Ph 10	A1	Bovine	LRT	Pneumonic	6.0	95 (0.6)	96 (0.3)
Ph 12	A1	Bovine	LRT	Pneumonic	8.0	83 (2.4)	90 (0.7)
Ph 14	A1	Bovine	LRT	Pneumonic	8.0	6 (0.6)	20 (2.7)
Ph 26	A1	Bovine	NP	Healthy	8.0	84 (1.6)	90 (0.4)
Ph 30	A1	Bovine	NP	Healthy	5.3	50 (0.4)	75 (1.5)
Ph 6	A1	Bovine	NP	Healthy	6.0	0 (0.1)	0 (0.1)
V965B	A1	Ovine	Lung	Pneumonic	6.0	67 (1.6)	89 (0.6)
FA 1	A1	Ovine	Lung	Pneumonic	7.3	78 (1.0)	85 (0.9)
Ph 42	A2	Bovine	Lung	Pneumonic	8.0	6 (1.2)	26 (1.5)
Ph 44	A2	Bovine	NP	Healthy	8.3	42 (1.1)	56 (2.7)
B664	A2	Ovine	Lung	Pneumonic	8.0	5 (2.0)	22 (1.7)
T884	A2	Ovine	Lung	Pneumonic	8.0	61 (2.5)	85 (0.8)
Ph 142	A2	Ovine	Lung	Pneumonic	8.0	18 (1.8)	85 (0.5)
10630	A5	Ovine	Lung	Pneumonic	6.3	95 (0.6)	97 (0.2)
10632	A6	Ovine	Lung	Pneumonic	6.3	94 (0.8)	95 (0.4)
10634	A7	Ovine	Lung	Pneumonic	6.3	95 (0.4)	95 (0.4)
10636	A8	Ovine	Lung	Pneumonic	7.0	93 (0.3)	94 (0.3)
10639	A9	Ovine	Lung	Pneumonic	6.3	96 (0.2)	97 (0.4)
10642	A11	Ovine	NP	Healthy	5.0	45 (1.2)	60 (2.6)
10644	A12	Ovine	NP	Healthy	4.3	90 (0.4)	92 (0.9)
11302	A13	Ovine	Lung	Pneumonic	6.3	91 (0.4)	94 (0.4)
10640	A13	Ovine	NP	Healthy	5.3	90 (0.3)	90 (0.5)
11303	A14	Ovine	Lung	Pneumonic	5.3	91 (0.6)	94 (0.7)
Ph 706	A16	Ovine	n/k	n/k	6.0	92 (0.5)	94 (0.6)
FT 3	T3	Ovine	NP	n/k	6.0	15 (2.1)	32 (2.0)
FT 4	T4	Ovine	Lung	Pneumonic	4.3	36 (1.1)	40 (1.6)
Ph 252	T10	Ovine	n/k	n/k	4.3	20 (1.0)	35 (0.9)
10641	T15	Ovine	Liver/spleen	n/k	4.3	25 (1.7)	39 (2.4)
UT 2	UT	Bovine	Lung	Pneumonic	3.3	75 (0.4)	82 (0.5)
UT 3	UT	Bovine	Lung	Pneumonic	5.3	100	100
UT 23	UT	Bovine	Lung	Pneumonic	4.3	30 (1.0)	39 (0.6)
UT 27	UT	Bovine	Lung	Pneumonic	5.0	83 (3.9)	85 (0.9)

UT, untypable; LRT, lower respiratory tract; NP, nasopharynx; n/k, not known.

*Time of harvest of culture (end of log phase) for assay of Lkt in culture supernate.

†Percentage inhibition of the normal CL response of neutrophils to opsonised zymosan (OZ). Neutrophils were incubated with culture supernate samples for 30 min at 37°C before addition of OZ. Results are mean values from five independent assays.

Stock cultures were stored at -70°C in Brain Heart Infusion Broth (BHIB; Oxoid) containing glycerol 50% v/v. The bacteria were grown routinely in BHIB or on BHI agar (BHIA; Oxoid) containing defibrinated sheep blood 5% v/v and incubated overnight at 37°C . All strains were characterised by the API 20 NE assay (API) and the serotypes of the A1, A2 and T strains were confirmed by indirect haemagglutination [19] with type-specific sera kindly provided by Dr W. Donachie.

Preparation of leukotoxin

Culture supernates, as a source of leukotoxin, were prepared from different isolates at different times during growth. Bacteria from overnight BHIB cultures at 37°C were washed and suspended in PBS. They were adjusted to an optical density of 0.4 at 640 nm and 1-ml volumes were inoculated into 50-ml volumes of BHIB in 250-ml conical shake flasks and incubated at 37°C and 120 rpm. Changes in optical density were monitored and, at intervals, cultures were centrifuged at 7000 *g* for 10 min at 4°C . The resulting supernates were passed through 0.45- μm membrane filters (Acrodisc; Gelman Sciences) and stored at -70°C .

For preparation of leukotoxin under different conditions of aeration, the growth conditions of Davies *et al.* [18] were used (i.e., very high aeration, high aeration, moderate aeration, low aeration, no aeration and CO_2 atmosphere).

Chemiluminescence (CL) assay

Heparinised blood was obtained from healthy calves and sheep. For preparation of neutrophils, 10-ml volumes of blood were diluted with equal volumes of PBS (pH 7.38) and carefully layered on top of 5 ml of Histopaque (Sigma) at a density of 1.077 g/ml and centrifuged 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 , 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 were washed twice with PBS and finally suspended in Hanks's HEPES (HH), containing (g/L): NaCl, 8; KCl, 0.4; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.19; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2; glucose, 1; HEPES 2.3, pH 7.38. The resulting cell suspension was > 95% neutrophils, and neutrophil viability, determined by trypan blue dye 0.1% w/v exclusion, was > 98% in all preparations examined.

For preparation of opsonised zymosan (OZ), 100 mg of zymosan A (Sigma; 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 OZ was washed twice with ice-cold PBS, pH 7.38, and finally suspended in 10 ml of PBS and stored at -20°C until used.

DNDH (7-dimethylamino-naphthalene-1, 2 dicarbonic acid hydrazide; Boehringer), was prepared as a 0.01 M stock solution in dimethyl sulphoxide. The working dilution (0.0001 M) was prepared in HH.

For the routine CL assay 15 μl of culture supernate containing leukotoxin were added to 885 μl of HH containing neutrophils and DNDH and incubated for 30 min at 38°C . Neutrophils were then stimulated with 100 μl of OZ and the resulting CL was measured with a Wallac luminometer connected to an IBM-pc computer with Multi-Use software (Bio-Orbit, Turku, Finland). The CL emission was measured in millivolts (mV) at 38°C . Each sample was tested in duplicate, and contained neutrophils (5×10^5), DNDH (10^{-5} M), OZ (0.5 mg), HH (pH 7.38) in a final volume of 1 ml. Results for leukotoxic activity are expressed as percentage inhibition of the peak value of the normal CL response of the neutrophils to OZ and are mean values from five independent assays.

Antibody preparation

For preparation of rabbit antibody to Lkt, recombinant Lkt (rLkt) expressed in *E. coli* and partially purified from insoluble inclusion bodies (G. D. Westrop *et al.*, unpublished report) was resolved by SDS-PAGE with acrylamide gel 7.5% w/v. The gel was stained with Coomassie Blue R 250 0.1% w/v for 5 min and then destained for 20 min. The band at 105 kDa was excised carefully, washed in sterile PBS, crushed, suspended in sterile PBS and dialysed at 4°C for 18 h against saline. The supernate from the dialysis sac was emulsified in an equal volume of Freund's incomplete adjuvant (Sigma). A female New Zealand White rabbit was inoculated intramuscularly with a total volume of 2 ml. Before injection of sample, the rabbit was bled for pre-immune serum. The final antiserum was collected 1 week after the rabbit had received four booster injections at monthly intervals.

Mouse hybridoma culture supernate containing monoclonal antibody (MAb) raised against rLkt from *E. coli* was kindly provided by Dr W. Donachie.

Analytical SDS-PAGE and immunoblotting

SDS-PAGE was done according to the method of Laemmli [20] in a vertical slab gel apparatus (Protean II; BioRad). Stacking gels and resolving gels contained acrylamide at 4% w/v and 10% w/v, respectively. Samples were solubilised in an equal volume of sample buffer and heated in a water bath at 100°C for 5 min. After SDS-PAGE, the resolved protein bands were transferred to a nitrocellulose membrane (Hybond-C

super, Amersham) by the method of Towbin *et al.* [21] in a BioRad transblot cell. Blots were probed with MAb to rLkt followed by anti-mouse IgG-HRP conjugate (Scottish Antibody Production Unit: SAPU).

ELISA

The wells of flat-bottomed, microtitration plates (Dynatech Immulon™) were coated with 350 µl of MAb to rLkt diluted 1 in 100 in 0.05 M carbonate buffer, pH 9.6. The plates were incubated at 4°C overnight in a humidified box then washed three times with washing buffer containing gelatin 0.5% w/v and Tween 20 0.01% v/v in PBS, pH 7.4. Culture supernate samples or rLkt were diluted in washing buffer and 300 µl were added to the wells. The plates were incubated for 2 h at 37°C in a humidified box and then washed as above. To each well, 250 µl of rabbit polyclonal antibody raised against rLkt, at a dilution of 1 in 3000 in washing buffer, were added. The plates were incubated for 4 h at 37°C in a humidified box, washed, and 250 µl of anti-rabbit IgG-HRP conjugate (SAPU) diluted 1 in 3000 in washing buffer were added to each well and the plate was incubated overnight at 4°C and washed as above. The enzyme reaction was initiated by the addition of 200 µl of *o*-phenylenediamine (34 mg/ml; Sigma) and H₂O₂ (20 µl) in 100 ml of 0.15 M citrate-phosphate buffer, pH 5.0 (freshly prepared). The plate was incubated for 30 min at room temperature in the dark for colour development. The reaction was stopped by addition of 50 µl of H₂SO₄ 12.5% v/v to each well. Absorbance was measured at 492 nm in an Anthos 2001 ELISA reader. As a reference preparation, a culture supernate from strain Ph 2, was included in each plate. This was assigned an arbitrary value of 100 Lkt units/ml. The Lkt antigen content of other samples was calculated with a parallel line assay by comparison of absorbance values for the reference and test samples.

Protein estimation

Protein content was estimated by the method of Bradford [22] with bovine serum albumin (Sigma) as a standard.

Results

Optimum conditions for Lkt production

Thirty-three *P. haemolytica* isolates, including representatives of all different serotypes and some untypable strains, were examined for Lkt production in culture supernates. In initial experiments, optimal conditions for growth and Lkt production in BHIB were determined. Growth of strains Ph 2 and Ph 30 under various conditions was monitored by spectrophotometry. Lkt production was judged by SDS-PAGE and immunoblotting of culture supernate samples, and Lkt activity was measured by the CL-inhibition assay.

When different conditions of aeration of the culture (i.e., very high aeration, high aeration, moderate aeration, low aeration, no aeration and CO₂ 5% atmosphere [18]) were examined, the maximum yield of toxin and growth was obtained with very high aeration, i.e., 50 ml of culture in a 250-ml dimpled-shake flask shaken at 120 rpm. With different temperatures (25°, 30°, 35°, 37°, 40° and 43°C) of incubation, *P. haemolytica* produced most toxin and grew optimally at 37°C.

The age of the culture had a major influence on Lkt yield, as judged by immunoblotting of culture supernate samples, and on Lkt activity, as measured by CL-inhibition with bovine neutrophils. As shown in Fig. 1, Lkt activity of strain Ph 2 growing in BHIB closely paralleled bacterial growth, with a maximum at the end of log phase. As the culture aged, there was a decline in toxin activity. Similar results were obtained with other *P. haemolytica* strains. Thus, in comparing Lkt activity of different strains of *P. haemolytica*, bacteria were grown in BHIB at 37°C with very high aeration and culture supernates were sampled at different times to include the end of log phase. The timing of this point of the growth curve varied somewhat from strain to strain (Table 1). Culture supernate samples containing Lkt showed a reduction in toxic activity on standing at room temperature or at 4°C and, to a lesser extent, even on prolonged storage at -70°C. Thus for comparative purposes, all preparations were tested immediately after harvest and a culture supernate sample from strain Ph 2 was used as control and internal standard in every assay.

CL inhibition by Lkt preparations

Results of a typical CL-inhibition assay, with bovine neutrophils and samples from three strains of *P. haemolytica*, are shown in Fig. 2. The culture supernate of strain Ph 2 was highly toxic and caused almost complete inhibition of CL, that from strain Ph 30 caused about 50% inhibition whereas that from strain B644 had little effect. Overall, culture supernate samples from most strains (21 of 33) were highly toxic (i.e., caused > 80% inhibition of the normal CL response) for bovine or ovine neutrophils, or both (Table 1). Samples from an untypable strain UT 3 proved to be the most toxic and gave 100% inhibition of CL even when diluted 1 in 5. Three strains were moderately toxic (41–80% inhibition) for bovine or ovine neutrophils, or both. Eight strains had low toxicity (< 40% inhibition) for both bovine and ovine neutrophils; these included one serotype A1, two A2, four biotype T (*P. trehalosi*) and one untypable strain. Only one strain, Ph 6, 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. Serial passage of *P. haemolytica* strains Ph 2 and Ph 10, up to 40 times, had no effect on the activity of Lkt produced.

Fig. 1. Lkt activity (—■—) in the culture supernate during growth (—○—) of *P. haemolytica* strain Ph 2 in BHIB. Lkt activity was measured by its inhibition of the normal CL response of bovine neutrophils to OZ.

Fig. 2. The effect of culture supernates from different strains of *P. haemolytica* on the CL response of bovine neutrophils to OZ: —●—, control CL response (no culture supernate added); —■—, CL response of neutrophils treated with culture supernate from strain Ph 2; —□—, strain Ph 30; —○—, strain B664.

By analysis of variance according to the general linear model (Minitab), the Lkt preparations from bovine isolates were, overall, significantly less toxic for neutrophils, as shown by inhibition of the normal CL response, than Lkt preparations from ovine isolates ($p = 0.018$). The mean percentage inhibition of CL was 61.91 (SEM = 3.04, $n = 130$) for bovine preparations and 70.36 (SEM = 2.11, $n = 200$) for ovine preparations. Ovine neutrophils were significantly ($p = 0.009$)

more sensitive to the Lkt preparations than bovine neutrophils. The mean percentage inhibition of CL was 71.86 (SEM = 2.27, $n = 165$) for ovine and 62.2 (SEM = 2.66, $n = 165$) for bovine neutrophils.

Specificity of the CL assay for Lkt

The specificity of the CL assay for Lkt was tested in various ways.

Culture supernate samples from *P. multocida* NCTC10322, presumably containing cell products common to *Pasteurella* spp., caused no inhibition of the CL response of bovine neutrophils to OZ.

Active rLkt, produced from the cloned *lktA* and *lktC* genes co-expressed in *E. coli* (G. D. Westrop *et al.*, unpublished report) completely inhibited the CL response of bovine neutrophils whereas non-activated Lkt, produced in the absence of LktC, had no effect. The rLkt preparations were tested at the same protein concentrations (3.5–35 µg/ml) and the active and non-activated rLkt preparations contained similar amounts of Lkt antigen as judged by immunoblotting and ELISA.

P. haemolytica produces LPS in the culture supernate, as shown by Western blotting with rabbit polyclonal serum raised against LPS. To test the possibility that the toxicity of culture supernates for neutrophils was due to, or affected by, contaminating LPS, additional studies were done. CL inhibitory activity was destroyed by boiling for 30 min (LPS is heat stable and would be expected to withstand this treatment). In addition, purified LPS (rough and smooth) from *P. haemolytica* A1 and LPS from smooth *E. coli* (kindly provided by Dr R. Davies) at concentrations up to 40 µg/ml had no significant inhibitory effect on the CL response of bovine neutrophils and did not kill the cells.

Finally, the CL-inhibitory activity was shown to be neutralised by antibody specific for Lkt. Culture supernates of *P. haemolytica* Ph 2 (undiluted) and active rLkt (1 in 1000 dilution) from *E. coli* were incubated for 30 min with polyclonal antiserum raised against purified rLkt and their toxicity was then measured in the CL assay. The antiserum completely inhibited the effect of both preparations of Lkt on the neutrophils.

Variation in amount and mol. wt of Lkt produced by different strains of *P. haemolytica*

All isolates produced Lkt in culture supernates, except Ph 6 which did not produce Lkt as judged by SDS-PAGE and immunoblotting with MAb. There was some variation in the mol. wt of Lkt produced by different strains of *P. haemolytica*. Most isolates (28 of 32) produced Lkt of *c.* 105 kDa, as shown in lanes 1, 3, 5, 7 and 9 (Fig. 3). Four strains (Ph 42, Ph 44, 10634, UT 23; lanes 2, 4, 6 and 8, respectively) produced Lkt of *c.* 108 kDa.

Fig. 3 and Table 1 also show that there was no clear relationship between the mol. wt and the amount of Lkt protein in the culture supernate, as judged by immunoblotting, and leukotoxic activity. The culture supernate samples in lanes 1, 5, 6 and 7 were highly toxic in the CL assay, that in lane 4 was moderately toxic whereas those in lanes 2, 3 and 8 had low toxicity but appeared to contain as much Lkt protein as the more toxic samples. This was confirmed by ELISA (Table 2). For example, the sample from isolate Ph 42 contained more of the 105 kDa Lkt protein than strain Ph 2, as determined by ELISA

Table 2. Measurement of Lkt by ELISA

Lkt preparation from strain no.	Amount of Lkt antigen (ELISA units/ml)	Lkt activity: percentage inhibition of CL response of neutrophils	
		Bovine	Ovine
Ph 2	100	97	97
Ph 42	150	6	26
Ph 14	84	6	20
FA 1	74	78	85
10634	81	95	95
UT 3	<10	100	100
UT 23	12	30	39

Fig. 3. Differences in mol. wt of Lkt proteins of *P. haemolytica* strains. Culture supernates from different strains, grown to late log phase in BHIB, were subjected to SDS-PAGE and immunoblotting with a MAb to Lkt. Lane 1, strain Ph 2; 2, Ph 42; 3, Ph 14; 4, Ph 44; 5, FA 1; 6, 10634; 7, UT 3; 8, UT 23; 9, UT 2. Positions of mol. wt markers are shown on the left.

(150 and 100 Units of Lkt antigen/ml, respectively) but the former had little leukotoxic activity on bovine and ovine neutrophils, whereas the latter was highly toxic. However, there were some discrepancies between the amounts of Lkt antigen detected by immunoblotting and by ELISA, as seen with the UT 3 and UT 23 preparations. Bands are clearly visible in the immunoblots (Fig. 3, lanes 7 and 8, respectively) and yet little antigen was detected by ELISA.

Discussion

The present study was designed to investigate whether different strains of *P. haemolytica*, used previously in this laboratory and which showed variation in LPS and OMP profiles [13, 14], produced different amounts of Lkt activity. To compare Lkt production by different strains, it was first necessary to standardise the growth and sampling conditions, as various factors such as temperature, pH, composition of growth medium, growth rate and time of harvest have been reported to influence Lkt activity [23, 24]. Lkt has been shown to be produced by actively growing cells and the level of toxicity in the culture supernate peaks at the end of log phase or earlier, depending on the growth medium [25, 26]. In the present study, with BHIB as the growth medium, growth rates varied from strain to strain but all strains produced maximum toxin activity at the end of log phase and thereafter the activity declined. Loss of 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.* [7], but the samples taken at the end of log phase 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.

Strains representative of serotypes 1–12 of *P. haemolytica* have been shown to produce leukotoxin activity, although some were reported to have only low toxicity [27–30]. Some untypable strains were found not to produce Lkt, but these were isolated from chickens rather than ruminants and so their identity may be in doubt [28]. In the present study, representatives of most of the 16 recognised serotypes and four bovine untypable strains were found to produce leukotoxic activity against bovine and ovine neutrophils. Of the 33 strains tested, most (24) were highly or moderately toxic. All four of the biotype T (*P. trehalosi*) strains had low toxicity, as did one serotype A1, two A2 and one untypable strain. The low toxicity of a T3 strain has been reported previously [31]. In the present panel of strains, nine A1 and five 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 OMP and LPS profiles in a similar panel of *P. haemolytica* strains [13–15]. Only one strain, Ph 6,

produced no detectable toxic activity, although Southern blot analysis showed that it contained the *lkt* genetic determinant (data not shown). This strain is serotype A1 but is atypical in other respects such as its biochemical, OMP and LPS profiles [14] and plasmid profile [16].

A surprising finding in the present study was that there was no clear relationship between the amount of Lkt 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 Ph 14 and 42 contained the 105-kDa Lkt protein in amounts similar to that from strain Ph 2 but had little leukotoxic activity. Such differences in toxicity of the Lkt preparations might be due to intrinsic differences in the Lkt proteins produced by the various strains or due to strain differences in other *Pasteurella* products such as the LktC activator protein or LPS. For example, it has been suggested that LPS may have a role in the haemolytic activity of *E. coli* haemolysin [32] and mutations in LPS biosynthesis have been shown to be responsible for reduction in haemolysin activity [33]. There is inter-strain variation in the LPS profiles of our *P. haemolytica* isolates [13] but there was no obvious correlation between Lkt activity and LPS type. Furthermore, neither smooth nor rough LPS from *P. haemolytica* alone caused any significant inhibition of the CL response of bovine neutrophils but a synergic effect with Lkt cannot be excluded.

Most strains produced Lkt of *c.* 105 kDa but four strains, (Ph 42, Ph 44, 10634 and UT 23) produced a higher mol. wt (*c.* 108 kDa) form. However, there was no obvious relationship between these larger Lkts and their toxic activities or the characteristics of the producing strain (Table 1). Thus, Ph 42 is a bovine A2 isolate from pneumonic lung and its culture supernate had low toxicity; Ph 44 is a bovine A2 strain but from the nasopharynx of a healthy animal and the sample had moderate toxicity; 10634 is an ovine A7 isolate from a pneumonic lung and its culture supernate was highly toxic and a bold Lkt band was evident in the immunoblot; and UT 23 is an untypable bovine lung isolate and had low toxicity.

Differences in mol. wt and toxicity of the leukotoxins from different serotypes have been reported previously although in most studies only a single strain of each serotype was examined [29, 30, 34, 35]. Lkt produced by a serotype T3 strain was significantly less toxic for bovine leucocytes than Lkt from serotype A1 and had a slightly higher mol. wt [31]. Recently, Burrows *et al.* [35] showed by SDS-PAGE and immunoblotting that reference strains of all 16 serotypes produced Lkt proteins that were antigenically related and had similar, but not identical, mol. wts. Other workers have demonstrated some antigenic differences in the Lkt proteins from certain serotypes by antibody

neutralisation tests [27, 30] and genetic differences in the *lkt* determinants have been revealed by Southern blotting with the serotype 1 *lkt* determinant as a probe [35]. In this laboratory, Southern blot analysis of selected strains with a probe incorporating the serotype-1 *lkt* determinant revealed restriction fragment length polymorphisms between different strains even within the same serotype (unpublished data). Antigenic differences in the leukotoxin proteins could account for the apparent discrepancies between the amounts of some of the Lkt antigens detected by immunoblotting and by ELISA. With preparations from strains UT 3 and UT 23, 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 was homologous, although both antibodies gave similar results in immunoblotting.

These data, taken together with our previous findings showing differences in LPS, OMP and plasmid profiles, even within a given serotype, reveal considerable heterogeneity in *P. haemolytica* isolates. Further analysis of the *lkt* genetic determinants of these strains may reveal that different genes coding for distinct leukotoxin proteins may exist within *P. haemolytica*, as has been found in *Actinobacillus pleuropneumoniae* [36]. Attempts to correlate these differences with virulence of the strains may prove useful in epidemiological studies.

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