Characterization of epitopes involved in the neutralization of *Pasteurella haemolytica* serotype A1 leukotoxin

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Defined segments of the leukotoxin A gene (*lktA*) from an A1 serotype of *Pasteurella haemolytica* were cloned into a plasmid vector and expressed as LacZ α fusion proteins. These fusion proteins were electrophoresed in SDS-PAGE gels and their immunoblotting reactivities with several monoclonal antibodies characterized. The epitope recognized by a strongly neutralizing monoclonal antibody was localized to a 32 amino acid region near the C terminus of the leukotoxin A (LktA) molecule. The epitope recognized by a non-neutralizing antibody was localized to a 33 amino acid region immediately adjacent. Smaller recombinant peptides containing these epitopes were not antigenic, but a polypeptide encompassing 229 amino acids at the C terminus evoked neutralizing antibodies when used to immunize specific-pathogen-free lambs. The distributions of linear epitopes recognized by this antiserum and by antisera raised to full-length recombinant LktA and to native LktA produced by *P. haemolytica* serotype A1 were determined by their reactivities with a set of overlapping 10 amino acid synthetic peptides. This revealed a complex distribution of linear epitopes at the C-terminal end of LktA. Toxin-neutralizing antibodies in convalescent sheep serum were shown to be directed against conformational epitopes by selective absorption of antibodies directed against linear epitopes.

Keywords: *Pasteurella haemolytica*, epitope, leukotoxin A, RTX toxin

INTRODUCTION

Pasteurella haemolytica is a major respiratory pathogen of ruminants but, because it can exist as a commensal, it is frequently isolated from the upper respiratory tract of apparently healthy sheep and cattle. Although the conditions which cause it to become an invasive pathogen are not known, several virulence factors have been identified, including proteins involved in iron acquisition (Donachie & Gilmour, 1988; Ogunnariwo & Schryvers, 1990; Gilmour *et al.*, 1991), outer membrane proteins (Donachie *et al.*, 1984), lipopolysaccharide (Fenwick, 1990), capsular polysaccharide (Czuprynski *et al.*, 1991a) and leukotoxin (LktA) (Lo *et al.*, 1987).

Abbreviations: *lktA*(A1) and *lktA*(T10), leukotoxin A genes from *Pasteurella haemolytica* serotype A1 and *Pasteurella trehalosi* serotype T10, respectively; LktA(A1) and LktA(T10), leukotoxin A gene products from *P. haemolytica* serotype A1 and *P. trehalosi* serotype T10, respectively; SPF, specific-pathogen-free.

The GenBank accession number for the nucleotide sequence reported in this paper is Z26247.

LktA is a potent cytolytic toxin secreted by both *P. haemolytica* and a closely related species *Pasteurella trehalosi* (Sneath & Stevens, 1990). It is a member of the RTX (repeat in toxin) family of pore-forming cytotoxins (Lo, 1995) which have been linked with the virulence of other bacterial species, for example *Escherichia coli* (Rowe *et al.*, 1994) and *Actinobacillus* spp. (Frey, 1995). *P. haemolytica* LktA is specific for ruminant leukocytes and is considered to be an important factor in establishing infection in the lung. It is believed to act *in vivo* by depleting the lung macrophage and neutrophil populations (Wilkie *et al.*, 1990; Breider *et al.*, 1991). Exposure to LktA also induces the release of oxygen-derived free radicals and proteolytic enzymes from neutrophils (Czuprynski *et al.*, 1991b; Maheswaran *et al.*, 1992) and histamine from bovine pulmonary mast cells (Adusu *et al.*, 1994) which may contribute to a localized inflammatory response and tissue damage. *In vitro*, the cytolytic effects of LktA on bovine leukocytes can be abrogated by LktA-neutralizing antibodies, indicating that antibodies which neutralize LktA may provide a degree of protection against infection. The role of LktA in protective immunity is supported by

successful vaccination and challenge experiments using specific-pathogen-free (SPF) lambs (Sutherland *et al.*, 1989) and calves (Shewen *et al.*, 1988; Conlon *et al.*, 1991). The antigenic similarity between the toxins produced by different serotypes of *P. haemolytica* results in the production of cross-neutralizing antibodies (Shewen & Wilkie, 1983). In ovine pasteurellosis this cross-neutralizing effect offers an antigenic advantage in vaccine formulation where a range of serotypes are involved (Fraser *et al.*, 1982).

Monoclonal antibodies with *in vitro* neutralizing activity against *P. haemolytica* LktA and the related toxin, α -haemolysin, in *E. coli* have been reported (Gentry & Srikumaran, 1991; Gerbig *et al.*, 1992; Pellett *et al.*, 1990). Monoclonal antibodies directed against a number of epitopes in the LktA molecule can differ widely in their capacity to neutralize LktA (Gerbig *et al.*, 1992) suggesting that domains are present in the toxin which are important in antibody-mediated neutralization and that intact LktA may not be required to evoke a neutralizing antibody response. Vaccination with an antigen that contains defined epitopes would ensure that an immune response would be produced only to epitopes which are relevant to protective immunity and would thus allow experimental evaluation of the efficacy of defined recombinant polypeptides of LktA as vaccine components. For pasteurellosis of sheep, such an approach may also permit incorporation of several epitopes from a range of serotypes which are relevant to the disease in sheep (Gilmour & Gilmour, 1989).

In the present study we investigated the epitopic structure of the C terminus of recombinant LktA and determined the ability of defined recombinant polypeptides derived from LktA to evoke production of toxin-neutralizing antibodies and hence their potential role as vaccine components.

METHODS

Strains and bacterial growth. *P. haemolytica* serotype A1 (strain SA1) and *P. haemolytica* serotype A2 (strain X205A) isolated from cases of ovine pneumonia, and *P. trehalosi* serotype T10 (strain 152/92) isolated from a case of systemic ovine pasteurellosis, were passaged on sheep blood agar and then stored at -70°C . A 1 ml aliquot of frozen cells was grown at 37°C for 18 h in 50 ml nutrient broth no. 2 (Gibco). This was used as seed for a 1 l culture in nutrient broth no. 2 grown in a 2 l flask for 5 h at 37°C on a rotary shaker. Bacterial cells were harvested by centrifugation at 10000 g and the pellets washed twice in PBS (0.15 M NaCl/0.01 M sodium phosphate, pH 7.4).

DNA manipulations. Genetic manipulations were carried out as described by Sambrook *et al.* (1989).

Cloning of restriction fragments of the *lktA* gene from *P. haemolytica* serotype A1 [lktA(A1)]. A series of constructs were made in which restriction fragments of plasmid pLkt52 (Lo *et al.*, 1987) were subcloned into the multiple cloning site (MCS) of pUC8 for inducible expression of LktA or defined recombinant polypeptides derived from LktA (Fig. 1). Plasmid pAL2 contains a 3938 bp *Bgl*II fragment of pLkt52 (Fig. 1) cloned into the *Bam*HI site of pUC8. In the remaining constructs, restriction fragments of the *lktA* gene were cloned

into the pUC8 MCS for IPTG-inducible expression as fusion proteins with the LacZ peptide.

In pAL4, an *Mae*I fragment of the *lktA* gene was isolated from construct pAL2, its blunt ends filled in using Klenow DNA polymerase I and then subcloned into the *Sal*I site of pUC8 which was similarly filled in. Plasmids pAL6 and pAL7 were deletants prepared from pAL2. For pAL6, pAL2 was digested with *Pst*I which cuts at two sites, one within *lktA* gene and the other in the pUC8 MCS; this construct was then ligated to circularize it. For construction of pAL7, pAL2 was digested at the *Eco*RV site within the *lktA* gene and at the *Hind*III site within the MCS of pUC8; the blunt ends were filled in using Klenow polymerase and the construct ligated. For pAL12, an *Eco*RV-*Hind*III fragment of pAL4 was isolated and then subcloned into the compatible *Hinc*II and *Hind*III sites of pUC8. For pAL14, a *Clal*-*Hind*III fragment of pAL4 was subcloned into the compatible *Acl*I and *Hind*III sites of pUC8. Cloning and expression were as carried out in *E. coli* strain JM109.

Cloning of PCR-generated *lktA* gene segments. Defined segments of *lktA* DNA were generated by PCR using pAL2 template DNA. Oligonucleotide primers were constructed on the basis of the published sequence data of Lo *et al.* (1987) and Highlander *et al.* (1989). The primers (forward/reverse) used in each construct were: pAL27, 478M/479M; pAL28, 763M/479M; pAL29, 764M/479M; pAL32, 888A/311J; pAL34, 764M/222P; pAL35, 764M/311J; pAL36, 764M/973P. The nucleotide sequences of these oligonucleotides are shown in Table 1. Restriction sites were engineered into the oligonucleotide primers to create an upstream *Eco*RI site in correct reading-frame alignment with the *lacZ'* gene start codon in pUC8 and a downstream *Hind*III site in correct reading-frame alignment with the alpha-fragment-encoding *lacZ'* ORF. The PCR products were cloned into the *Eco*RI and *Hind*III sites of pUC8 to produce a series of $\Phi(lktA'-lacZ')$ (Hyb) derivatives and DNA sequence analyses carried out to confirm the identity of inserts. LktA'-LacZ α fusion proteins were expressed in *E. coli* strain JM109 from the IPTG-inducible *lac* promoter of pUC8. pAL32 made use of an upstream *Eco*RV site located within the *lktA* sequence (Fig. 1). The *Eco*RV-*Hind*III fragment was then cloned into the compatible *Hinc*II and *Hind*III sites in pUC8.

Expression of recombinant LktA. For expression, cultures were grown to OD₆₀₀ 0.5 and then induced for 3 h with 0.5 mM IPTG. Cells were harvested by centrifugation, washed twice in PBS, solubilized in SDS-PAGE sample buffer and then analysed by SDS-PAGE using 12% (w/v) polyacrylamide gels and immunoblotting.

Cloning of the *lktA* gene from *P. trehalosi* serotype T10 [lktA(T10)]. The *lktA* gene was isolated from a T10 serotype genomic DNA library constructed in the vector lambda EMBL3 using a *lktA*(A1) pAL2 insert as a DNA probe. The nucleotide sequence of the *lktA*(T10) gene was determined using T7 sequencing reagents (Pharmacia) and oligonucleotide primers (Oswel DNA Services).

SDS-PAGE and immunoblotting. Recombinant LktA and LktA'-LacZ α fusion proteins were analysed by SDS-PAGE on 12% polyacrylamide gels using prestained low-molecular-mass size standards from BioRad. Proteins were transferred electrophoretically onto Immobilon-P membranes (Millipore) using the method of Towbin *et al.* (1979). After blocking binding sites, the membranes were probed with monoclonal or polyclonal antibodies diluted to previously optimized concentrations. The appropriate species-specific anti-immunoglobulin, conjugated to horseradish peroxidase, was used according to the

Table 1. PCR oligonucleotide primers used for generating defined DNA segments of *lktA* for subcloning

Primer	Direction	Location*	Nucleotide sequence†
478M	Forward	2682–2710	AAAAGTGAATTCTAACTATGAATTGCTCA
763M	Forward	2482–2508	ACGAAGAATTCAAAGAGAAAGTGACC
764M	Forward	2362–2388	CACCGGAATTCGATGGTAATGATATT
888A	Forward	467–488	GATTTAGATGAGGCCTTACA
479M	Reverse	2839–2865	AAAATAAGCTTGTCTAGCAAAATGAA
311J	Reverse	2503–2534	ATCAGCCAAGCTTAACCAGTTTTGAATGGTCA
222P	Reverse	2700–2729	GTCACAAAGCTTCTATGTTTGAGCAATCA
973P	Reverse	2602–2630	ACTTGCAAGCTTGTGATCCGCTCGCCATT

* The location of oligonucleotides within the *lktA* gene (2859 bp) are shown (Lo *et al.*, 1987). Nucleotide numbering begins with the first nucleotide of the start codon of *lktA*.

† Sequences are shown from 5' to 3' ends; underlining shows the position of engineered restriction sites, *Eco*RI for forward primers and *Hind*III for reverse primers.

supplier's directions and the immunoblot developed using the substrate diaminobenzidine (Sigma).

Purification of recombinant LktA(A1) from *E. coli* strain JM109 containing plasmid pAL2. *E. coli* cells expressing pAL2-encoded LktA(A1) were disrupted by sonication and centrifuged at 20000 *g* at 4 °C. The resulting pellet (the LktA-enriched fraction) was solubilized in 2% (w/v) SDS and the solubilized material fractionated by gel filtration on Sephacryl S300 in PBS containing 0.1% SDS. Fractions containing the 105 kDa protein were pooled. The protein was concentrated and further purified by absorption on to a hydroxyapatite Bio-Gel HTP (BioRad) column in 10 mM sodium phosphate buffer, pH 7.0 containing 0.1% SDS and elution with a linear gradient of buffered phosphate from 0 to 0.5 M, pH 6.8, containing 0.1% SDS. Eluates were monitored by SDS-PAGE and fractions containing almost pure 105 kDa protein were pooled. SDS was removed by exhaustive dialysis against 50 mM Tris/HCl buffer, pH 7.6, containing 1.0 mM PMSF. The resulting LktA(A1) was > 90% pure as determined by SDS-PAGE and immunoblots and contained 0.2 mg protein ml⁻¹.

Monoclonal antibodies. Ascitic fluids containing monoclonal antibody (mAb) MM601, which is strongly neutralizing and mAb MM605, which is non-neutralizing towards native LktA(A1) (Gentry & Srikumaran, 1991), were obtained from Dr M. Gentry, University of Nebraska, Nebraska, USA.

Six monoclonal antibodies were produced in mice against recombinant LktA(A1) expressed from plasmid pAL2. Mice were immunized intraperitoneally with 25 µg purified recombinant LktA in Freund's complete adjuvant and boosted 4 weeks later with the same preparation in Freund's incomplete adjuvant. Three days prior to hybridoma fusion two mice were boosted intravenously with 10 µg immunizing antigen. For fusion, cells from homogenized spleens were fused with the mouse plasmacytoma SP-2 line in the presence of PEG according to the method of Kohler & Milstein (1975). Wells containing hybridomas were screened for specific antibody against native LktA by ELISA. Cells secreting reactive antibody were then cloned by limiting dilution and the mAbs designated JM1 to JM6. Expression of anti-LktA antibody was confirmed by immunoblot analysis using recombinant LktA expressed from pAL2.

Polyclonal antibodies. SPF lamb antiserum to pAL2-encoded LktA was prepared by inoculating two 3-week-old SPF lambs

intramuscularly with 100 µg purified LktA antigen in 1 ml Al(OH)₃ on two occasions, 4 weeks apart. Serum was taken from the lambs before immunization and after the second vaccination.

Recombinant LktA'-LacZα fusion proteins expressed by pAL12, pAL34 and pAL36 were excised from polyacrylamide gels after SDS-PAGE separation and the identity of the excised fusion polypeptide confirmed by immunoblot analysis using mAb MM601. For inoculation, this material was treated as described by Murray *et al.* (1992). pAL12-encoded antigen was used to immunize SPF lambs, and pAL34- and pAL36-encoded antigens were used to immunize rabbits.

Antiserum S791 was raised in SPF lambs by aerosol administration of *P. haemolytica* serotype A2. Sera were taken from surviving lambs 3 weeks later and pooled. This was regarded as convalescent antiserum. Antiserum SA1 was raised in a conventionally reared sheep with no anti-LktA titre. The sheep was inoculated subcutaneously with washed *P. haemolytica* serotype A1 cells from a 6 h culture in nutrient broth. The sheep was bled 2 weeks after inoculation and the serum stored at -20 °C.

LktA cytotoxicity assay and neutralization test. Cell culture supernatant fraction from serotype A1 cells in early exponential phase (1–3 h) in RPMI medium containing either 7% (v/v) foetal bovine serum (FBS) or 5 mg BSA ml⁻¹ was filtered through a 0.22 µm membrane, dialysed and freeze-dried in 1 ml aliquots. Stock toxin was prepared by dissolving freeze-dried material in Hank's phosphate buffered saline (PBS) containing 7% FBS and mixed with either sheep macrophages or bovine lymphoma (BL3) cells at 10⁷ cells ml⁻¹ in Hank's PBS at room temperature for 1 h. Cytotoxicity was measured by a Trypan blue exclusion test in which the percentage of dead cells was estimated by microscopic examination. For neutralization tests, toxin was mixed with doubling dilutions of antibody for 15 min at room temperature and then added to the target cells for 1 h. Lysis of cells was again tested by the Trypan blue exclusion test. An alternative cytotoxicity test (Cell Titer 96, Promega) was used to monitor the absorption of antisera. This test is based on the reduction of a soluble tetrazolium dye by BL3 cells in Hank's PBS, FBS at room temperature. Dye reduction was monitored at A₄₉₀.

Antibody absorption. Lyophilized LktA-enriched fraction (0.5 mg) of an *E. coli* (pAL2) lysate was suspended in 100 µl 10 mM Tris/HCl buffer, pH 7.0, containing 5% SDS and 5%

(v/v) β -mercaptoethanol and then heated to 100 °C. This solution was diluted to 10 ml in 10 mM Tris/HCl, pH 7.0 and the antigen adsorbed onto Immobilon-P membranes (Millipore) at room temperature with shaking for 2 h. The efficiency of adsorption was monitored by an immunoblot reaction with mAb MM601 using a section of the membrane. The membrane was washed extensively in 10 mM Tris/HCl buffer, pH 7.5, containing 0.05% Tween. Strips of approximately 7 cm² were used to absorb antibodies from 10 ml aliquots of SA1 and S791 antisera, respectively, each diluted 1/40 in PBS. The immunoblotting reaction with recombinant Lkt antigen and the neutralizing capacity of both antisera were determined before absorption and after each of four sequential absorptions carried out at room temperature.

Peptide synthesis and epitope screening. Peptides were synthesized on a solid phase using a modification of the method of Geysen *et al.* (1987). Reagents were supplied by Cambridge Research Biochemicals. Overlapping synthetic peptides were constructed on the basis of the published sequence data for *P. haemolytica* serotype A1 (Lo *et al.*, 1987). The region of LktA investigated extended from amino acid 722 to the C terminus of LktA, amino acid 953. Synthesized peptides were 10 amino acids long and the start point for each successive peptide was progressed by 3 amino acid residues. Duplicate peptides were screened with various antisera using an ELISA with horseradish peroxidase conjugated to various anti-species immunoglobulin. Absorbance was measured at 405 nm.

RESULTS

Characteristics of anti-LktA antibodies

In tests using the Trypan blue method to determine neutralizing titres, mAb MM601 had a neutralizing titre of > 1/4000, whereas mAb MM605 failed to neutralize at

a dilution of 1/10. mAbs JM1–JM6 gave strong immunoblotting reactions at a dilution of 1/40 but no neutralization could be detected in undiluted hybridoma supernatants. The polyclonal antibodies S791 and SA1 had neutralizing titres of 1/400 and 1/4000, respectively.

Epitope mapping reveals the position of an epitope concerned with toxin neutralization

Defined segments of the *lktA*(A1) gene were subcloned in pUC8 for expression of LktA-derived polypeptides as LacZ α fusion proteins. The reactivities of these expressed proteins with various antibodies were determined by immunoblotting (Fig. 1). The epitopes recognized by neutralizing mAb MM601 and non-neutralizing mAb MM605, and also by the six monoclonal antibodies JM1–JM6 (data not shown), all lay within a 229 amino acid region (722–950) at the C-terminal end of LktA. This is defined by the positive reaction of these mAbs with the LktA-derived product expressed from plasmid pAL12. More detailed mapping of this region relied on cloning and expression of DNA subclones generated by PCR (Fig. 2). The epitope for mAb MM601 was mapped to a 32 amino acid region on the basis of its reaction with LktA-derived polypeptides expressed from plasmids pAL36 and pAL28, but not with those expressed from plasmids pAL35 or pAL32. The epitope for MM605 was mapped to a 33 amino acid region immediately downstream on the basis of its reaction with the LktA-derived polypeptide expressed from pAL34 but not with that expressed from pAL36. Controls included in the immunoblotting procedure showed that none of the mAbs used reacted with

Fig. 1. Immunoblot reactions of mAbs MM601 and MM605 with LktA(A1) and LktA(A1)-derived polypeptides expressed from *lktA* subclones in pUC8 constructs in *E. coli*. The filled box represents the ORF for the *lktA* gene. Each construct is shown, together with the amino acid region spanned by the expressed LktA(A1)-derived polypeptide and its size. Positive (+) and negative (–) reactions are indicated.

Fig. 2. Immunoblot reactions of mAbs MM601 and MM605 with LktA(A1)-derived polypeptides encoded by PCR-generated DNA segments of *lktA* cloned into pUC8 and expressed in *E. coli*. Each plasmid construct is shown together with the amino acid region spanned by the expressed LktA-derived polypeptide and its size. Positive (+) and negative (–) reactions are indicated.

E. coli lysates or with the LacZ α component of the fusion protein.

Comparison with LktA(T10)

The mAbs MM601 and MM605 did not react with LktA(T10) on immunoblots and did not neutralize LktA(T10) *in vitro*. This lack of cross-reactivity is probably due to amino acid sequence differences within the linear epitopes recognized by these antibodies between the LktAs produced by A1 and T10 serotype strains. We cloned and determined the nucleotide sequence of the *lktA* gene from a T10 serotype strain of *P. trehalosi*. The deduced amino acid sequences of the C-terminal 205 amino acids of A1 and T10 serotypes are shown in Fig. 3. The positions of the epitopes recognized by the mAbs MM601 and MM605, as determined by the mapping experiments described above, are indicated. Comparison of these amino acid sequences confirmed that differences exist in the regions of both epitopes and further localized the A1 epitopes to positions where the amino acid sequences of the A1 and T10 toxins differed.

Antigenicity of expressed LktA(A1)-derived recombinant polypeptides

E. coli(pAL2) expressed the full-length LktA(A1) molecule (953 amino acids) in a biologically inactive form. *E. coli* cells bearing pAL12 expressed a LktA'–LacZ α fusion protein which encompassed 229 amino acids at the

C-terminal end of LktA(A1) (Fig. 1). After partial purification by gel filtration, these proteins were used to immunize SPF lambs. The antisera obtained were strongly reactive against full-length recombinant LktA on immunoblots and had a LktA-neutralizing titre of > 256 as determined by the Trypan blue method. *E. coli* bearing plasmids pAL34 and pAL36 (Fig. 2) expressed smaller LktA' peptides as fusion proteins which encompassed the epitopes recognized by both mAb MM601 (neutralizing) and mAb MM605 (non-neutralizing) and by mAb MM601 alone, respectively. Bands containing these fusion peptides, which had been excised from SDS-PAGE gels, were used to immunize rabbits but neither fusion peptide elicited an antibody response that could be detected by immunoblot analysis using pAL2- or pAL12-encoded antigen.

Epitope mapping of the C terminus of LktA using synthetic peptides

mAb MM601 did not react with any of the 75 10 amino acid synthetic peptides produced on a solid support. However, various antisera directed against LktA(A1) did react, and identified several potential linear B-cell epitopes located near the C terminus of LktA (Fig. 4). Although relative peak heights were different, similar profiles were obtained for each of the antisera tested, namely, polyclonal antiserum raised in an SPF lamb against the C-terminal fragment of recombinant LktA(A1) expressed from plasmid pAL12 (anti-LktA') (Fig. 4b), polyclonal anti-

749	RLFGGKGDDI	LDGGNGDDFI	DGGKGNDLLH	GGKGDDIFVH	RKGDGNDIIT	DSDGNDKLSF	SDSNLKDLTF	EKVKHNLVIT
	I				Q	S	E E	N H
829	NSKKEKVTIQ	NWFREADFAK	EVPNYKATKD	EKIEEIIQGN	GERITSKQVD	DLIAKGNGKI	TQDELSKVVD	NYELLKHSKN
	T Q	E	TIQ V R D			E E	A S T	Q Y RD
	Neutralizing mAb M601				Non-neutralizing mAb MM605			
909	VTNSLDKLIS	SVSAFTSSND	SRNVLVAPTS	MLDQSLSSLQ	FARAA*			
	AS	A	AS	P I				

Fig. 3. Comparison of the amino acid sequences (residues 749–953) of LktA(A1) and LktA(T10). The LktA(A1) sequence is shown in full; where the LktA(T10) sequence differs, the substituted amino acid is shown. The location of epitopes for the neutralizing (MM601) and the non-neutralizing (MM605) mAbs are indicated. The C terminus of LktA is indicated by an asterisk.

Fig. 4. Epitope mapping of the C terminus of LktA(A1) encompassing amino acid residues 722–953. A set of 75 overlapping 10 amino acid peptides was synthesized on a solid phase. The sequences of individual peptides are shown in (a). The positions of epitopes for mAbs MM601 and MM605 as defined in Fig. 3 are indicated by * and †, respectively. The reactivities of these peptides in ELISA with three antisera are shown in (b)–(d). (b) SPF lamb anti-LktA' (C-terminal polypeptide-LacZα fusion protein encoded by pAL12), (c) SPF lamb anti-LktA(A1) encoded by pAL2 and (d) sheep anti-A1 live cells.

serum raised in an SPF lamb against the full-length recombinant LktA(A1) expressed from plasmid pAL2 (anti-LktA) (Fig. 4c) and sheep SA1 antiserum developed

after an active infection with *P. haemolytica* serotype A1 (Fig. 4d). SPF lamb pre-immune sera and a conjugate control produced no significant reaction with these

Table 2. Neutralizing titres of absorbed and non-absorbed polyclonal antisera SA1 or S791 and the strength of reactions with recombinant LktA (pAL2 product) by immunoblotting

	SA1 antiserum		S791 antiserum	
	Neutralizing titre*	Immunoblot reactivity†	Neutralizing titre*	Immunoblot reactivity†
Pre-immune	—	—	—	—
Pre-absorption	1280	++++	80	++++
Absorption 1	NT	++++	NT	++++
Absorption 2	640	++	80	++
Absorption 3	640	+/-	80	+/-
Absorption 4	640	—	80	+/-

* NT, Not tested.

† The strength of reaction on immunoblots was graded from nil (—) to very strong (++++).

peptides. The deduced positions of the epitope for mAb MM601 (peptides 39–50) and mAb MM605 (peptides 49–61) are indicated (Fig. 4a).

Antibodies with specificity for non-linear epitopes are identified in convalescent antisera

Denatured full-length recombinant LktA(A1) immobilized on a solid phase was used to absorb out specific antibodies from a homologous antiserum SA1 (anti-A1 serotype antiserum in response to infection) and from a heterologous antiserum S791 (anti-A2 serotype antiserum in response to infection). The results of absorptions of these SA1 and S791 antisera were similar (Table 2).

The antibodies directed against the linear epitopes in these antisera were quantified by their reaction with denatured recombinant LktA(A1) on immunoblots. The amounts of these antibodies were significantly reduced by the absorption procedure to the point that an immunoblotting reaction could not be detected in absorbed antiserum at 1/40 dilution. However, antibody absorptions did not produce proportional reductions in their LktA-neutralizing capacities, which was reduced by approximately 50% of the non-absorbed antiserum level in the case of SA1 antiserum and was unaffected for the S791 antiserum. Neutralization capacity found in absorbed antiserum was attributed to the presence of LktA-neutralizing antibodies directed against non-linear epitopes. Neutralization due to non-specific factors present in the antisera were not indicated since S791 and SA1 pre-immune sera did not react with LktA(A1) on immunoblots and had no toxin-neutralizing capacities.

DISCUSSION

These results showed that both linear and conformational epitopes within LktA(A1) are involved in antibody-mediated toxin neutralization and that a defined fragment of LktA(A1) is a potential vaccine component.

Vaccination experiments have established that crude preparations of native LktA can play an important role in protective immunity to ovine pasteurellosis (Sutherland *et al.*, 1989) and bovine pasteurellosis (Shewen *et al.*, 1988; Rice-Conlon *et al.*, 1995) and that a purified recombinant LktA can significantly enhance the protective capacity of a vaccine containing other cellular components (Conlon *et al.*, 1991). A positive correlation has also been demonstrated between serum anti-LktA antibody titres and protective immunity (Mosier *et al.*, 1986, 1989) and between toxin-neutralizing antibody titre and protective immunity (Gentry *et al.*, 1985; Sutherland *et al.*, 1989; Rice-Conlon *et al.*, 1995). Thus, antibody-mediated toxin neutralization provides a plausible means by which the pathogenicity of *P. haemolytica* may be reduced *in vivo*.

Gentry & Srikumaran (1991) and Gerbig *et al.* (1992) demonstrated that a mAb can neutralize LktA(A1) *in vitro*. Pellett *et al.* (1990) also demonstrated that a structurally related RTX toxin, α -haemolysin of *E. coli*, could be neutralized by an anti-LktA monoclonal antibody. Hence, the possibility arises that an LktA-derived peptide containing either a single epitope or a limited set of epitopes might be an effective component of a *P. haemolytica* vaccine.

The location of an epitope recognized by an LktA-neutralizing antibody

The monoclonal antibodies described here were from two independent cell fusions and were raised against either recombinant LktA (JM1–JM6) or native LktA (mAbs MM601 and MM605; Gentry & Srikumaran (1991). Each of these antibodies reacted with full-length recombinant LktA(A1) and with defined polypeptides derived from LktA(A1) indicating that their epitopes lay within a 229 amino acid region at the C-terminal end of the toxin. Since these monoclonal antibodies recognized denatured LktA(A1) on immunoblotting, they are probably specific for linear sequences of amino acids.

Although the mAbs JM1–JM6 reacted strongly with this

C-terminal polypeptide of LktA(A1), they showed no neutralizing activity. This demonstrates that there are antigenic regions within the polypeptide which are not targets for neutralizing antibodies. More detailed mapping with the two mAbs raised to native toxin localized the epitope for the strongly neutralizing mAb to a 32 amino acid region near the C-terminus of LktA(A1) (residues 841–872) and that for the non-neutralizing mAb to a 33 amino acid region immediately adjacent (residues 873–905). The close proximity of epitopes for two mAbs with distinctly different neutralizing capacities supports a model, such as that proposed by Forestier & Welch (1991), for the existence of distinct functional domains within the *P. haemolytica* LktA(A1) molecule. An attempt to map the location of the epitope of the neutralizing mAb at higher resolution by scanning a series of synthetic peptides was unsuccessful because the mAb failed to react. The reason for the failure is unclear but may reside in the specificity of the mAb for a spatial arrangement in the epitope which cannot be reproduced in a short synthetic peptide. However, a more precise location of this epitope could be deduced from comparison of the amino acid sequences of LktA(T10) and LktA(A1). Since the neutralizing mAb reacted with LktA(A1) but not LktA(T10) the epitope must lie in the region where the amino acid sequences differ, for example residues 845 to 859. Gerbig *et al.* (1992) similarly localized an epitope for a neutralizing mAb to the C-terminal region of LktA(A1). This epitope was implicated in target cell recognition or binding.

Immunogenicity of recombinant LktA(A1)-derived polypeptides

The presence of a neutralizing epitope in the C-terminal region of Lkt(A1) led us to examine its immunogenic properties and potential as a vaccine component. Full-length recombinant LktA(A1) (expressed from plasmid pAL2) and a recombinant LktA(A1)-derived polypeptide (expressed from plasmid pAL12) both evoked antibody responses in SPF lambs with LktA-neutralizing activity. By reacting these antisera with the same set of synthetic peptides described above, it was possible to determine the distribution of reactive epitopes within the C-terminal region of LktA(A1). Both antisera produced a complex distribution pattern indicating that several epitopes lie in this region. The pattern produced by these antisera and by an antiserum (SA1) obtained by exposure of a sheep to live cells of *P. haemolytica* serotype A1 corresponded closely. Thus, the route of exposure and form in which the antigen was presented did not greatly affect the profile of the antibody response to the linear epitopes present at the C-terminus of LktA.

Among this set of linear epitopes it was not possible to determine which are specifically involved in neutralization. However, it was possible to identify an epitope in a position corresponding to the epitope defined by the toxin-neutralizing mAb.

Mapping epitopes involved in antibody-mediated toxin neutralization on the basis of examining the immunological response to defined peptides proved to be im-

practical; small LktA-derived recombinant polypeptides expressed from plasmids pAL34 and pAL36, which contained the neutralizing epitope were found not to be immunogenic in rabbits. Further investigation of methods of antigen presentation of these peptides is warranted.

Are conformational epitopes involved in neutralization of LktA?

In a natural infection with *P. haemolytica* it is likely that the host immune system produces antibodies which are directed against both linear and non-linear epitopes of LktA. We have been able to directly identify the distribution of linear epitopes; however, it is more difficult to locate conformational epitopes and epitopes generated by post-translational modification of LktA(A1) and to determine their role in antibody-mediated neutralization.

Whilst the form of antigen presentation does not appear to have a marked effect on the immunological response to linear epitopes at the C terminus of LktA, it is likely that this will greatly affect the response to conformational epitopes. For example, during nasal carriage or natural infection with *P. haemolytica*, the host animal may be exposed to LktA in its native conformation for an extended period of time. In contrast, vaccination with recombinant or denatured LktA may produce antibodies which are predominantly directed against linear epitopes, although the extent to which the molecule can spontaneously refold into its native configuration in a vaccine is unknown.

We used antibody absorption to selectively remove the antibodies directed against linear epitopes from polyclonal antisera and then quantified the LktA-neutralizing capacity of the remaining antibodies, which presumably react with non-linear epitopes. This showed that in a natural infection antibodies are produced against linear and non-linear epitopes and that both can play a role in toxin neutralization. Whether a single epitope mediates neutralization *in vivo* or whether neutralization requires concerted interaction with several epitopes is presently unknown and should be examined in any future work.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Mr K. Aitchison, and the financial support of the Scottish Office Agriculture, Environment and Fisheries Department and Hoechst Animal Health, UK in this work.

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Received 30 August 1995; revised 25 April 1996; accepted 7 May 1996.