MANNHEIMIA HAEMOLYTICA LEUKOTOXIN – HOST CELL RECEPTOR INTERACTIONS

By

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MANNHEIMIA HAEMOLYTICA LEUKOTOXIN – HOST CELL RECEPTOR INTERACTIONS

Abstract

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Mannheimia haemolytica is the primary bacterial pathogen of bovine pneumonic pasteurellosis, an economically important disease of cattle worldwide. Leukotoxin (Lkt) produced by *M. haemolytica* is the major virulence factor of this organism. The cytolytic activity of Lkt is specific for ruminant leukocytes. Lkt utilizes CD18, the β subunit of β_2-integrins, as its receptor on ruminant leukocytes. Previously, our laboratory mapped the Lkt-binding domain to lie between amino acids (aa) 1-291 of CD18. Therefore, the next logical step was to identify the precise Lkt binding site within this domain and to determine whether co-administration of CD18 peptide analogs would inhibit / mitigate *M. haemolytica*-caused lung injury.

In this study, by using synthetic peptides spanning aa1-291 of bovine CD18 in Lkt-induced cytolysis assays, the precise binding site of Lkt was mapped to aa 5-17 of ruminant CD18. Surprisingly, all the aa of this peptide belong to the predicted signal peptide of CD18. This observation led to the finding that the signal peptide of ruminant CD18 is not cleaved, and that the intact signal peptide renders ruminants susceptible to *M. haemolytica* Lkt. Site-directed mutagenesis of a single aa in the signal peptide resulted in the cleavage of signal peptide and abrogation of Lkt-induced cytolysis of target cells. This finding indicates that engineering cattle

and other ruminants to contain this mutation would provide a novel technology to render them less susceptible to pneumonic pasteurellosis and concomitant economic losses.

The peptide spanning aa 5-17 (P17) was used in a calf challenge study which was designed as a 'proof of concept' experiment. Even though the difference in percent volume of lungs exhibiting gross pneumonic lesions between P17-inoculated calves and control peptide-inoculated calves was not statistically significant, *M. haemolytica* isolated from the lungs of P17-inoculated calves was 100- to 1000-fold less than those from the control peptide-inoculated calves, suggesting that P17 reduced leukotoxic activity in the lungs which enhanced bacterial clearance by phagocytes. It is likely that prolonging the presence and activity of CD18 peptide analog in the lungs, for example by means of a nanoparticle delivery system such as dextran nanospheres, would enhance its protective activity.

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Mannheimia haemolytica leukotoxin – host cell receptor interactions

GENERAL INTRODUCTION

Bovine pneumonic pasteurellosis, more commonly known as shipping fever, is an economically significant respiratory disease of both beef and dairy cattle industry in North America and Western Europe (Mosier, 1997; Ames, 1997). The annual economic losses to the US cattle industry have been estimated to be as high as $1 billion (Bowland and Shewen, 2000). *Mannheimia haemolytica* is the primary bacterial agent responsible for the pathophysiological events leading to this acute fibrinonecrotizing pneumonia (Whiteley et al., 1992). *M. haemolytica* is commonly found as a commensal bacterium in the upper respiratory tract of healthy cattle. Exposure of cattle to stress factors or viral or other bacterial infections leads to proliferation of *M. haemolytica* in the upper respiratory tract. Once present in high levels, it enters the alveolar spaces through repeated aspiration of infected droplets and sloughed cells/tissues. Here, it initiates an inflammatory cascade, causing pneumonia with the massive neutrophil influx along with accumulation of fibrin and subsequent necrosis in the alveolar spaces. *M. haemolytica* has been isolated worldwide, but the prevalence of disease strongly correlates with Western animal management practices that include overcrowding and transport. The organism can also cause pneumonic and septic disease in other ruminants, including domestic sheep (Filion et al., 1984), goats (Msra et al., 1970), bighorn sheep (Foreyt et al., 1994, Dassanayake et al., 2009), and bison (Dyer and Ward, 1998).

M. haemolytica produces several virulence factors which include the capsule, outer membrane proteins, adhesins, neuraminidase, lipopolysaccharide and leukotoxin (Lkt; Confer et al., 1990). Of these virulence factors, Lkt has been accepted as the most important one based on the observations that Lkt-deletion mutants induce much milder lung lesions and reduced mortality

than the wild-type organisms (Petras et al., 1995; Tatum et al., 1998; Highlander et al., 2000; Dassanayake et al., 2009). Lkt produced by *M. haemolytica* is a member of the RTX (repeats in toxin) family of toxins produced by a number of Gram-negative bacteria. Toxins of the RTX family lyse their target cells primarily through formation of pores (Coote, 1992) which leads to the efflux of K^+, influx of Ca^{2+}, colloidal osmotic swelling and eventual cell lysis (Clinkenbeard et al., 1989). The cytolytic activity of *M. haemolytica* Lkt is specific for ruminant leukocytes (Kaehler et al., 1980; Shewen and Wilkie, 1982; Chang et al., 1986). While all subsets of leukocytes are susceptible to Lkt-induced cytolysis, polymorphonuclear leukocytes (PMNs) are the most susceptible subset. Moreover, PMN depletion in calves has been shown to drastically reduce the lung lesions. Lysis of alveolar macrophages and PMNs impairs the phagocytic ability of the host which facilitates proliferation and survival of bacteria within the lung. Cytolysis of these cells also results in the release of their proteolytic enzymes and pro-inflammatory substances, which cause structural degradation of the alveolar epitheilial linings. Therefore, Lkt-induced lysis and de-granulation of the alveolar macrophages and PMNs are responsible for the acute inflammation and lung injury characteristic of this disease.

Previously, our laboratory and that of others have independently shown that the cytotoxic effect of Lkt on bovine and ovine leukocytes is mediated by Lkt-β_2-integrin interactions (Wang et al., 1998; Ambagala et al., 1999; Li et al., 1999). β_2-integrins are leukocyte-specific integrins that are critical for homing of leukocytes to the sites of inflammation, phagocytosis, antigen presentation, and cytotoxicity (Gamberg e al., 1998; Luscinskas et al., 1989). They are heterodimeric glycoproteins composed of α subunit (CD11), and β subunit (CD18). CD18 associates with four distinct α chains to give rise to four different β_2-integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (CR4), and CD11d/CD18 (Gahmberg et al., 1998;

and Noti et al., 2000). In previous studies in our laboratory, the recombinant expression of bovine or ovine CD18 in Lkt non-susceptible cell lines rendered them susceptible to Lkt-induced cytolysis, suggesting that CD18, the β subunit of β_2-integrins, serves as the receptor for Lkt on leukocytes (Deshpande et al., 2002; Liu et al., 2007; Dassanayake et al., 2007a). Monomeric expression of CD18 on a transfectant cell-line confirmed that CD18 is the functional receptor for Lkt (2007b). Subsequently, by constructing bovine-murine CD18 chimeras, the Lkt-binding site was mapped to a domain on CD18 encompassing amino acids 1 to 291 (Gopinath et al., 2005).

The next logical step, therefore, was to identify the precise binding site of Lkt within the CD18 domain encompassing amino acids 1-291, which formed the first objective of this study. Binding of a ligand to its receptor could potentially be inhibited by synthetic peptides representing the amino acids comprising the binding site of the ligand on the receptor (Tibetts et al., 1999, 2000). Therefore the first hypothesis of this study was that peptide analogs of bovine CD18 will inhibit the Lkt-induced cytolysis of bovine leukocytes.

Cytotoxicity assays with a nested set of peptides spanning amino acids 1 to 291 of CD18 identified the Lkt-binding domain to lie between the amino acids 5 to 17. Surprisingly, these amino acids comprise most of the amino acids from signal peptide of CD18, which suggested that the signal peptide of CD18 of cattle, and possibly other ruminants may not be cleaved. However, the paradigm dictates that the signal peptides of membrane proteins are cleaved once the nascent proteins reach the endoplasmic reticulum for the post-translational modifications. Further experiments were designed to answer the question as to whether the signal peptide of CD18 of ruminants remains intact on the mature CD18 molecule, and if so, whether the intact signal peptide renders ruminants susceptible to *M. haemolytica* Lkt.

The nucleotide and predicted amino acid sequence of CD18 of five ruminants were available in the GenBank (cattle, buffalo, domestic sheep, bighorn sheep and goat). In order to enhance the validity of theoretical observations that could be made, the cDNA encoding CD18 of bison, deer and elk were cloned. The molecular cloning of CD18 of bison, deer and elk, and their comparison with that of other ruminants and non-ruminants are described in the manuscript in Chapter 1 which is under review for publication in *Veterinary Immunology and Immunopathology*.

The results of the other experiments which determined that the signal peptide of CD18 of ruminants is indeed not cleaved, and that the intact signal peptide of CD18 renders the ruminants susceptible to *M. haemolytica* Lkt, are described in the manuscript in Chapter 2 which has been published in the *Proceedings of the National Academy of Science, USA* (Volume 106, pages 15448-15453).

The second hypothesis of this study was that the peptide analogs of CD18 will inhibit lung lesions caused by *M. haemolytica* in calves. The results of this 'proof of concept' study involving the endobronchial co-inoculation of a CD18 peptide analog with *M. haemolytica* are described in the manuscript in Chapter 3.

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CHAPTER ONE

Molecular cloning of CD18 of bison, deer and elk, and comparison with that of other ruminants and non-ruminants

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ABSTRACT

Pneumonia caused by *Mannheimia haemolytica* is an important disease of cattle, domestic sheep, bighorn sheep and goats. Leukotoxin (Lkt) produced by *M. haemolytica* is cytolytic to all leukocyte subsets of these species. Lkt utilizes CD18, the β subunit of β_2-integrins, as its functional receptor on leukocytes of these species. Cytotoxicity assays revealed that leukocytes from bison, deer, and elk are also susceptible to Lkt-induced cytolysis. The availability of cDNA encoding CD18 of bison, deer and elk would facilitate the comparison of a greater number of ruminant CD18 cDNA with that of non-ruminants as a means of elucidation of the molecular basis for the specificity of *M. haemolytica* Lkt for ruminant leukocytes. Herein, we report the cloning and characterization of bison, deer, and elk CD18. The full length cDNA of bison and deer consists of 2310 bp with an ORF encoding 769 amino acids while elk CD18 consists of 2313 bp with an ORF encoding 770 amino acids. This gene is highly conserved among ruminants compared with non-ruminants. Phylogenetic analysis based on amino acid sequences showed that CD18 of bison is most closely related to that of cattle while CD18 of deer and elk are more closely related to each other.

INTRODUCTION

Mannheimia (Pasteurella) haemolytica is the most important bacterial pathogen of respiratory disease in cattle, domestic sheep (DS), bighorn sheep (BHS), and other domestic and wild ruminants. *M. haemolytica* causes an acute fibrino-necrotic pleuropneumonia resulting in extensive economic losses world-wide (Ackermann and Brogden, 2000; Miller, 2001; Odugbo et al., 2004). This Gram-negative bacterium produces several virulence factors which include an exotoxin that is cytolytic to all subsets of leukocytes, and hence referred to as leukotoxin (Lkt). Based on the observation that Lkt-deletion mutants of *M. haemolytica* cause reduced mortality and much milder lung lesions than the wild-type organisms, Lkt has been accepted as the most important virulence factor (Petras et al., 1995; Tatum et al., 1998; Highlander et al., 2000; Dassanayake et al., 2009). Cytolytic activity of *M. haemolytica* Lkt is specific for ruminant leukocytes (Kaehler et al., 1980; Chang et al., 1986).

Earlier studies by us and others (Wang et al., 1998; Ambagala et al., 1999, Li et al., 1999; Jayaseelan et al., 2000) identified β_2-integrins as the receptor for Lkt of *M. haemolytica* on bovine leukocytes. β_2-integrins, expressed exclusively on leukocytes, are composed of two non-covalently associated subunits, α (CD11) and β (CD18). CD18 associates with four distinct α chains to give rise to four different β_2-integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (CR4), and CD11d/CD18. Studies in our laboratory have demonstrated that CD18, the β subunit of β_2-integrins, is necessary and sufficient to mediate Lkt-induced cytolysis of bovine and ovine leukocytes (Deshpande et al., 2002; Liu et al., 2007; Dassanayake et al., 2007a, 2007b). Furthermore, we have mapped the Lkt binding site on bovine CD18 to lie between amino acids 1-291 (Gopinath et al., 2005). The nucleotide and deduced amino acid sequence of CD18 of cattle, DS, BHS, goats, and buffalo have been determined (Shuster et al., 1992; Zechinon et al.,

2004a; Liu et al., 2006; Zechinon et al., 2004b). Availability of sequence information on CD18 of additional ruminant species would facilitate the comparison of a greater number of ruminant CD18 cDNAs with that of non-rumninants as a means of elucidation of the molecular basis underlying the specificity of *M. haemolytica* Lkt for ruminant leukocytes, which in turn should pave the way for the development of strategies to control this economically important disease in ruminants. Therefore the objective of this study was to clone and sequence the cDNA encoding the CD18 of bison (*Bison bison)*, deer (*Odocoileus hemionus*), and elk (*Cervus canadensis)*, and compare that with CD18 of other ruminants and non-ruminants.

MATERIALS AND METHODS

PMNs and PBMCs of bison, deer and elk were isolated from peripheral blood by Ficoll-Paque (Amersham, NJ) density gradient centrifugation as described previously (Deshpande et al., 2002). The susceptibility of these cells to *M. haemolytica* Lkt-mediated cytolysis was confirmed by a previously described cytotoxicity assay {MTT [3-(4,5-dimethylthiazoyl-2-Yl)-2,5-diphenyl tetrazolium bromide] dye reduction assay}(Ambagala et al., 1999).

The total RNA from PBMCs was extracted using TRIzol reagent and cDNA was synthesized using Superscript^{TM} III first-strand synthesis system for RT-PCR following the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). Forward and reverse primers to amplify the *CD18* gene of bison and deer were designed based on multiple alignment of human (NM_000211), mouse (X14951), pig (U13941), chimpanzee (NM_001034122), cattle (M81233), DS (AY484425), BHS (DQ104444), goat (AY452481) and Buffalo (AY842449) cDNA sequences available in the GenBank. The primers designed to amplify *CD18* cDNAs of bison and deer were: CD18 For; 5'-GGCATCCAGGGGACATGC-3' and CD18 Rev; 5'-

CCCCTAACTCTCGGCAAAC-3'. Gene was amplified using a high fidelity polymerase, PfuUltra^{TM}II fusion HS (Strategene, La Jolla, CA). Single band PCR amplicons were gel-purified and cloned into pCR^{R} 4 Blunt-TOPO vector (Invitrogen). Following transformation of TOP10 chemically competent cells, positive clones were selected on LB-ampicillin plates and the insert was identified by colony PCR. Plasmid DNA from selected positive colonies was isolated and purified with QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. A total of 5 clones were sequenced using BigDye Terminator Chemistries and an ABI Prism 377 DNA sequencer (Applied Biosystems, CA). The bison and deer CD18 cDNA sequences have been deposited at GenBank (accession no. EU553919 and EU623794, respectively).

The sequences of 5'- and 3'- untranslated regions (UTR) of elk CD18 were obtained by using a 2nd generation 5'/3' RACE kit (Roche applied Science, Germany). Gene-specific primers were designed based on the sequence alignment of cloned cattle, DS, BHS and mouse, human, and pig CD18. A gene-specific sense primer (5'-GACAACAGCTCCATCATCTGCTC-3') and an anti-sense primer (5'-GTCCTGGTCGCAAGTAAAGTGTC-3') were used to amplify the 3' and 5' ends of the elk CD18 cDNA, respectively, according to the manufacturer's instructions. The RACE amplicons were cloned into the pCR^{R} 4 Blunt-TOPO vector. The positive clones containing inserts were identified by colony PCR and sequenced completely. cDNA from total RNA was synthesized using Superscript^{TM} III first-strand synthesis system for RT-PCR following the manufacturer's instructions to obtain the full length CD18 coding sequences (CDS) using primers designed to amplify deer and bison CD18 cDNAs. Full length elk CD18 was cloned and sequenced by a protocol similar to that used for the CD18 of bison and deer, and the sequence was deposited at GenBank (accession no. EU553918).

DNA sequence analysis, fragment assembly, and amino acid sequence prediction were performed with the ContigExpress module of Vector NTI AdvanceTM 9.1 (Invitrogen). SignalP V.2.0b2 (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997) and NetNGlyc V.1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) (Jensen et al., 2002) provided signal peptide and N-glycosylation sites prediction, respectively. BLAST (http://www.ncbi.nlm.nih-gov/) was used for homology and % identity, and open reading frame (ORF) were confirmed by ExPASY (http://us.expasy.org). Alignment of nucleotide and amino acid sequences and similarity analyses were performed with CLUSTAL-W1.8 (http://www.ebi.ac.uk; Thompson et al., 1994). Protein statistics were analyzed by EMBOSS server (http://www.bioinformatics.wsu.edu/emboss/).

RESULTS AND DISCUSSION

Lkt lysed PMNs and PBMCs of bison, deer, and elk in a concentration-dependent manner, as observed with the leukocytes of other ruminants (Fig. 1). The Fig 2 shows the complete nucleotide and derived amino acid sequences of bison, deer, and elk CD18. The bison and deer CD18 cDNA contain an ORF of 2307 bp which codes for 769 amino acids. The deduced polypeptides were 95% identical to each other. Elk CD18 cDNA contains 2310 bp coding for 770 amino acids. The deduced amino acid sequence from the coding region of elk CD18 shows 95% and 97% identity with that of bison and deer, respectively. The predicted molecular masses of the CD18 of bison, deer, and elk are 84.3 kD, 84.5 kD, and 84.7 kD, respectively. The predicted isoelectric points of bison, deer, and elk CD18 proteins are 6.0, 7.4, and 7.2, respectively. The comparison of deduced amino acid sequence of bison, deer, and elk CD18 with that of cattle, goats, DS, BHS, buffalo, pigs, humans, chimpanzees, mice and rats is shown in Table 1. The

identity in amino acid sequence among the ruminants ranges from 94% to 99%. The identity in amino acid sequence between the ruminants and non-ruminants ranges from 81% to 88%.

All three cloned CD18 proteins have a predicted N-terminal signal peptide sequence of 22 amino acids (maximum probability of 0.963). The predicted transmembrane domain is 23 amino acids long, which is followed by a cytoplasmic domain of 46 amino acids. Both regions are 100% identical among ruminants. The overall protein structure and domains are in agreement with previously identified CD18 of other species. The extracellular domains of CD18 of bison, deer, and elk contain 4, 7, and 6 N-linked glycosylation sites (Asn-X-Ser/Thr), respectively (Fig. 2). As with the CD18 of other species, the extracellular domain of bison, deer and elk CD18 also consists of an I-like domain of 240 amino acids (from amino acid 126 to 365 in elk and from amino acid 124 to 363 in bison and deer; Fig. 3A). It is identical to that found in other ruminant CD18, and quite similar to that of the CD18 of non-ruminants sequenced to-date. The I-like domain contains a metal ion-dependent adhesion site (MIDAS, DLSYS; Fig. 3A). CD18 of bison, deer and elk also contain 56 cysteine residues at identical positions, possibly involved in the formation of disulfide bridges.

A phylogenetic tree was constructed with the Neighbor-joining algorithm (Saitou and Nei, 1987) included in the MEGA 4 program. Distance was estimated using the p-distance method. The robustness of the inferred trees was assessed by bootstrap (1000 replicates) analysis (Felsenstein, 1985). Fig 3B shows the phylogenetic relationship among the CD18 of different species. Deer and elk sequences are more closely related to each other compared to that of other species, and the bison sequence shows a closer relationship to that of cattle and then buffalo, than to CD18 from other species.

In summary, we have cloned and sequenced the cDNA encoding CD18 of bison, deer and elk. The deduced amino acid sequence of CD18 of bison exhibits 95% identity with that of deer and elk CD18, while the CD18 of deer and elk exhibit 97% identity with each other. The identity in amino acid sequence among the ruminants ranges from 94% to 99%. The identity in amino acid sequence between the ruminants and non-ruminants ranges from 81% to 88%.

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Table 1 Comparison of the amino acid sequence of the CD18 of bison, deer and elk with that of cattle, domestic sheep (DS), bighorn sheep (BHS), goats, buffalo, pigs, humans, chimpanzees (Chimp), mice and rats.

	Cattle	DS	BHS	Goat	Buffalo	Deer	Bison	Elk	Pig	Human	Chimp	Mouse
DS	**95**	**-**										
BHS	**95**	**99**	**-**									
Goat	**96**	**99**	**98**	**-**								
Buffalo	**98**	**95**	**95**	**96**	**-**							
Deer	**95**	**95**	**95**	**95**	**94**	**-**						
Bison	**99**	**96**	**95**	**96**	**98**	**95**	**-**					
Elk	**95**	**95**	**95**	**96**	**95**	**97**	**95**	**-**				
Pig	**88**	**88**	**87**	**88**	**88**	**88**	**88**	**88**	**-**			
Human	**83**	**83**	**82**	**83**	**83**	**84**	**83**	**84**	**83**	**-**		
Chimp	**83**	**83**	**83**	**83**	**83**	**84**	**83**	**84**	**83**	**99**	**-**	
Mouse	**81**	**81**	**80**	**81**	**81**	**81**	**81**	**81**	**80**	**81**	**81**	**-**
Rat	**81**	**81**	**81**	**81**	**81**	**81**	**81**	**81**	**80**	**81**	**81**	**81**

Figure 1

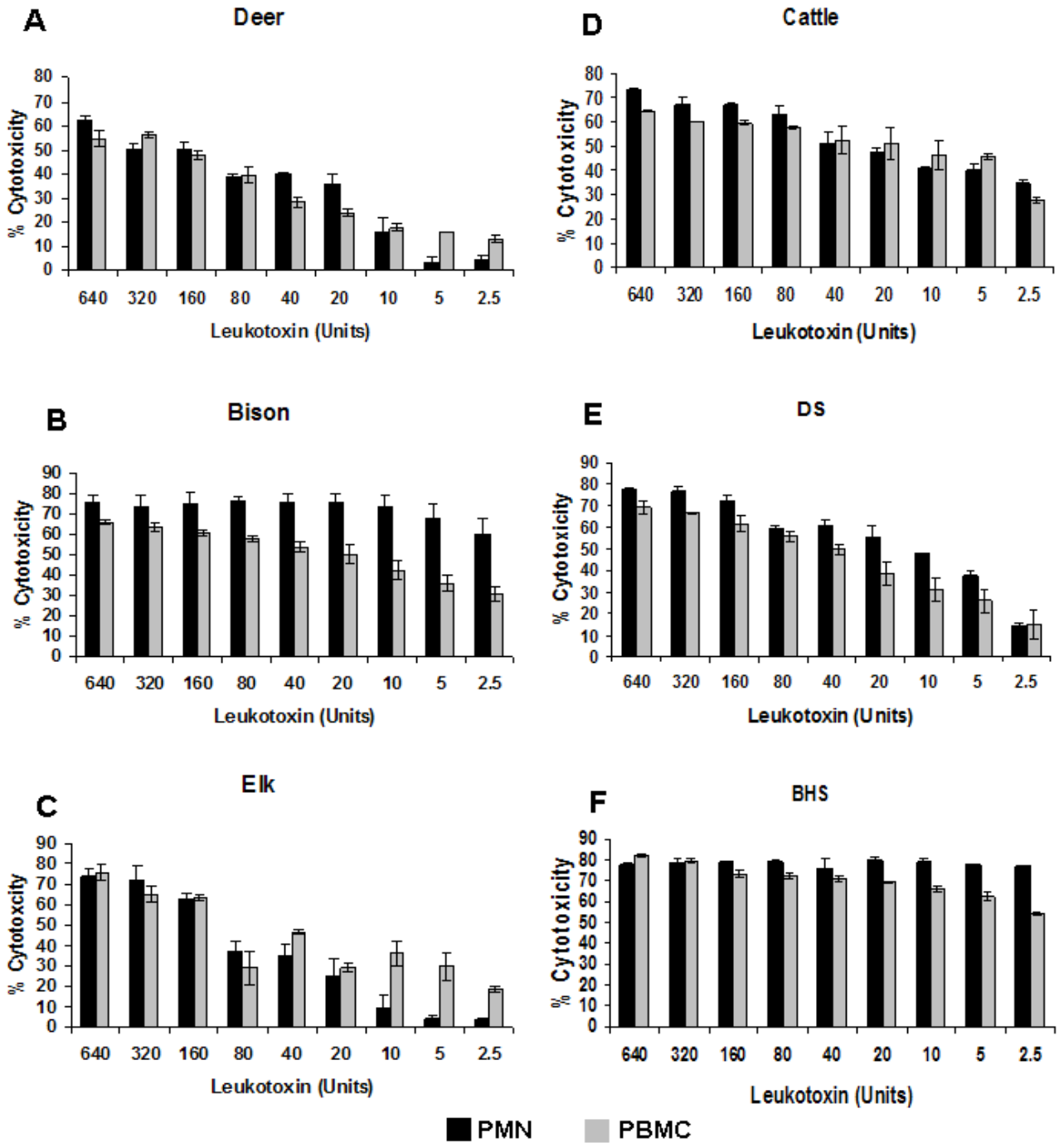
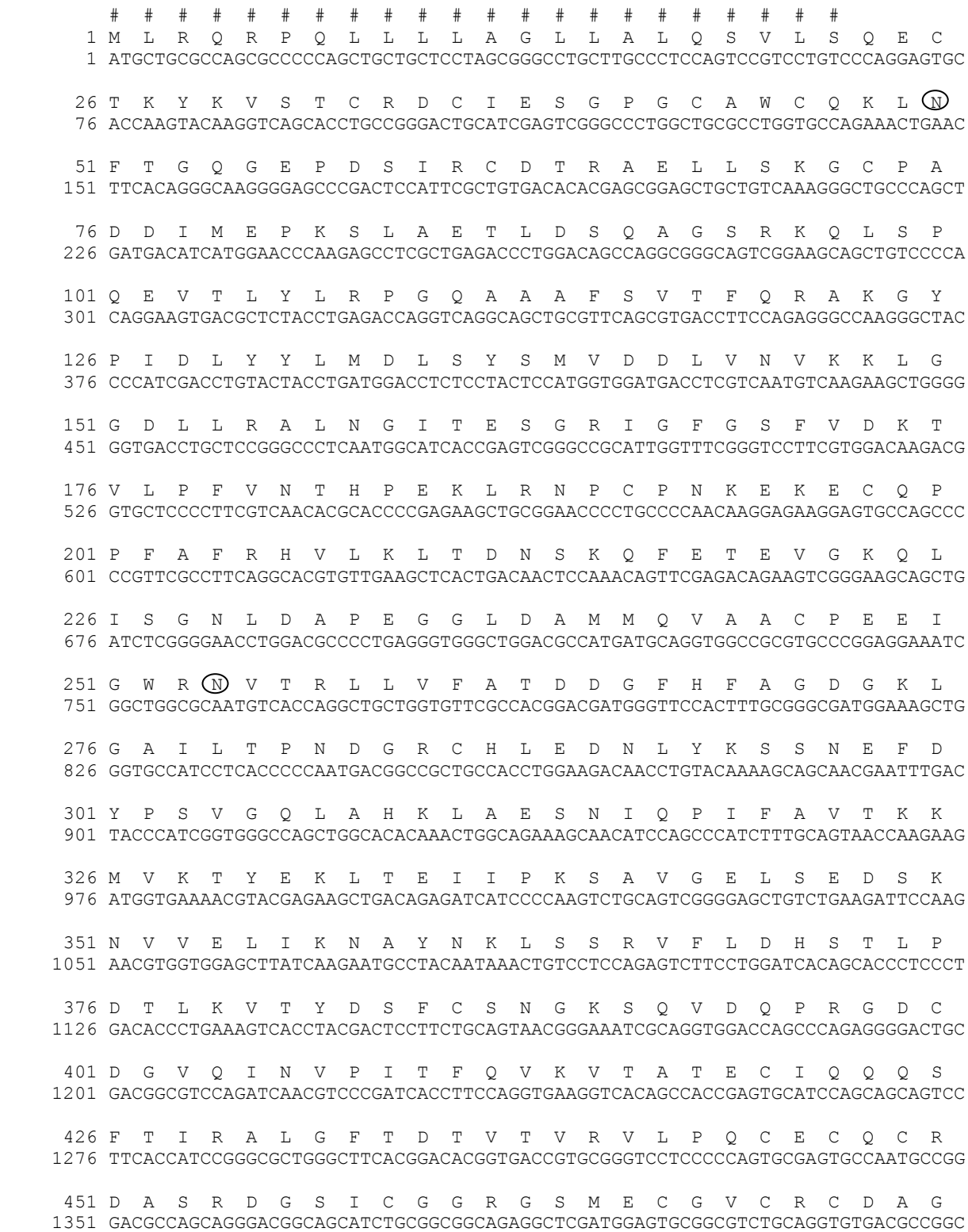

A
Deer
% Cytotoxicity
Leukotoxin (Units)
640 320 160 80 40 20 10 5 2.5
D
Cattle
B
Bison
E
DS
C
Elk
F
BHS
PMN
PBMC

Fig. 1. PMNs and PBMCs from bison, deer and elk are lysed by Lkt in a concentration-dependent manner similarly to those from cattle, domestic sheep, and bighorn sheep. The cells were tested for susceptibility to Lkt-induced cytolysis by the MTT dye-reduction cytotoxicity assay. The % cytotoxicity was determined using the formula: % cytotoxicity = [1-(OD of toxin-treated cells/OD of toxin-untreated cells)] X 100. Black bars represent the % cytotoxicity of PMNs and grey bars represent that of PBMCs. Results of one representative experiment out of three are shown.

Figure 2A


# #
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76 ACCAAGTACAAGGTCAGCACCTGCCGGGACTGCATCGAGTCGGGCCCTGGCTGCGCCTGGTGCCAGAAACTGAAC
51 F T G Q G E P D S I R C D T R A E L L S K G C P A
151 TTCACAGGGCAAGGGGAGCCCGACTCCATTCGCTGTGACACACGAGCGGAGCTGCTGTCAAAGGGCTGCCCAGCT
76 D D I M E P K S L A E T L D S Q A G S R K Q L S P
226 GATGACATCATGGAACCCAAGAGCCTCGCTGAGACCCTGGACAGCCAGGCGGGCAGTCGGAAGCAGCTGTCCCCA
101 Q E V T L Y L R P G Q A A A F S V T F Q R A K G Y
301 CAGGAAGTGACGCTCTACCTGAGACCAGGTCAGGCAGCTGCGTTCAGCGTGACCTTCCAGAGGGCCAAGGGCTAC
126 P I D L Y Y L M D L S Y S M V D D L V N V K K L G
376 CCCATCGACCTGTACTACCTGATGGACCTCTCCTACTCCATGGTGGATGACCTCGTCAATGTCAAGAAGCTGGGG
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526 GTGCTCCCCTTCGTCAACACGCACCCCGAGAAGCTGCGGAACCCCTGCCCCAACAAGGAGAAGGAGTGCCAGCCC
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```

Figure 2B

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 376 D  T  L  K  V  T  Y  D  S  F  C  S  N  G  V  S  K  V  D  Q  P  R  G  D  C
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 401 D  G  V  Q  I  N  V  P  I  T  F  Q  V  K  V  T  A  T  E  C  I  Q  E  Q  S
1201 GACGGCGTCCAGATCAACGTCCCGATCACCTTCCAGGTGAAGGTCACAGCCACCGAGTGCATCCAGGAGCAGTCC

 426 F  T  I  R  A  L  G  F  T  D  T  V  T  V  R  V  L  P  Q  C  E  C  Q  C  R
1276 TTCACCATCCGGGCCCTGGGCTTTACGGACACGGTGACCGTGCGGGTCCTCCCCCAGTGCGAGTGCCAATGCCGG

 451 D  A  S  R  D  R  S  V  C  G  G  R  G  S  M  E  C  G  V  C  R  C  D  A  G
1351 GACGCGAGCAGGGACCGCAGCGTCTGCGGTGGCAGAGGCTCGATGGAGTGCGGCGTCTGCAGGTGCGACGCCGGC

 476 Y  I  G  K  N  C  E  C  Q  T  Q  G  R  S  S  Q  E  L  E  G  S  C  R  K  D
1426 TACATCGGGAAGAACTGCGAGTGCCAGACGCAGGGCCGGAGCAGCCAGGAGCTGGAGGGCAGCTGCCGCAAGGAC

 501(N) S  S  I  I  C  S  G  L  G  D  C  I  C  G  Q  C  V  C  H  T  S  D  V  P
1501 AACAGCTCCATCATCTGCTCGGGGCTGGGGGACTGCATCTGCGGGCAGTGCGTGTGCCACACGAGCGACGTTCCC

 526 N  K  K  I  Y  G  Q  F  C  E  C  D  N  V  N  C  E  R  Y  D  G  Q  V  C  G
1576 AACAAGAAGATCTACGGCCAGTTCTGCGAGTGCGACAACGTCAACTGCGAGCGCTACGACGGCCAAGTCTGCGGG

 551 G  D  K  R  G  L  C  F  C  G  T  C  R  C  Q  D  Q  Y  E  G  S  A  C  Q  C
1651 GGCGACAAGAGGGGGCTCTGCTTCTGCGGCACCTGCAGGTGCCAGGACCAGTACGAGGGCTCGGCGTGCCAGTGC

 576 L  K  S  T  Q  G  C  L  N  L  N  G  V  E  C  S  G  R  G  R  C  R  C  N  V
1726 CTCAAGTCCACGCAGGGCTGCCTCAACCTGAACGGCGTCGAGTGCAGCGGCCGCGGCCGGTGCCGCTGCAACGTG

 601 C  Q  C  D  P  G  Y  Q  P  P  L  C  K  E  C  P  G  C  P  A  P  C  A  G  F
1801 TGCCAGTGCGACCCCGGCTACCAGCCGCCCCTGTGCAAAGAGTGCCCGGGCTGCCCCGCGCCCTGCGCCGGCTTT

 626 A  S  C  T  E  C  L  K  F  D  K  G  P  F  A  K (N) C  S  A  A  C  G  E  T
1876 GCCTCCTGCACCGAGTGCCTGAAGTTCGACAAGGGCCCCTTCGCCAAGAACTGCAGCGCAGCTTGCGGGGAGACG

 651 K  L  L  S  S  P  P  P  G  R  K  C  K  E  R  D  S  E  G  C  W  M  T  Y  T
1951 AAGCTGCTGTCCAGCCCGCCGCCCGGCCGCAAGTGCAAGGAGCGCGACTCCGAGGGCTGCTGGATGACCTACACC
```

```
 676 L  V  Q  R  D  G  R  D  R  Y  D  V  H  V  (N) D  T  R  E  C  V  K  G  P  N
2026 CTGGTGCAGCGCGACGGGCGGGACAGATACGACGTGCACGTGAACGACACGCGCGAGTGTGTAAAGGGCCCCAAC

 701 I  A  A  I  V  G  G  T  V  A  G  V  V  L  V  G  I  L  L  L  V  I  W  K  A
2101 ATCGCTGCCATTGTGGGGGGCACCGTGGCGGGAGTTGTGCTTGTCGGCATCCTCCTGCTGGTCATCTGGAAGGCC

 726 L  T  H  L  S  D  L  R  E  Y  H  R  F  E  K  E  K  L  K  S  Q  W  N  N  D
2176 CTGACACACCTGAGCGACCTCAGGGAGTACCATCGCTTCGAGAAGGAGAAGCTCAAGTCCCAGTGGAACAACGAT

 751 N  P  L  F  K  S  A  T  T  T  V  M  N  P  K  F  A  E  S  *
2251 AACCCTCTTTTCAAGAGTGCCACCACGACAGTCATGAACCCTAAGTTTGCCGAGAGTTAG
```

Figure 2C

```
-89 AAGCACCAGCCTGGTGAAGAGCAGAGCCGAAGCCCCTGCCAGTCCAGCCGGGACGTCCCTGCCGAGGTCTCCAGG

-14 GCATCCAGTGGGAC

    #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #
  1 M  L  R  Q  R  P  Q  L  L  L  L  A  G  L  L  A  L  Q  S  V  Q  S  Q  E  C
  1 ATGCTGCGCCAGCGCCCCCAGCTGCTGCTCCTAGCGGGCCTGCTAGCCCTCCAGTCTGTCCAGTCCCAGGAGTGC

 26 T  K  Y  K  V  S  T  C  R  D  C  I  E  S  G  P  G  C  A  W  C  Q  K  L  (N)
 76 ACCAAGTACAAGGTCAGCACCTGCCGGGACTGCATCGAGTCGGGCCCCGGCTGTGCCTGGTGCCAGAAGCTGAAC

 51 F  T  G  Q  G  E  P  D  S  A  R  C  D  T  R  A  Q  L  L  S  K  G  C  A  A
151 TTCACAGGGCAAGGGGAGCCCGACTCCGCTCGCTGTGACACACGGGCGCAGCTGCTGTCAAAGGGCTGTGCCGCT

 76 D  D  I  M  D  P  R  S  L  A  E  T  R  E  S  Q  A  G  R  Q  K  Q  K  Q  L
226 GATGACATCATGGATCCCAGGAGCCTCGCTGAGACCCGGGAGAGCCAGGCGGGCAGACAGAAGCAGAAGCAGCTG

101 S  P  Q  E  V  T  L  Y  L  R  P  G  Q  A  A  A  F  (N) V  T  F  Q  R  A  K
301 TCCCCACAGGAAGTGACGCTCTACCTGAGACCAGGTCAGGCAGCTGCGTTTAACGTGACTTTCCAGAGGGCCAAG

126 G  Y  P  I  D  L  Y  Y  L  M  D  L  S  Y  S  M  V  D  D  L  V  N  V  K  K
376 GGCTACCCCATCGACCTGTACTACCTGATGGACCTCTCCTACTCCATGGTGGATGACCTCGTCAACGTCAAGAAG

151 L  G  G  D  L  L  R  A  L  N  D  I  T  E  S  G  R  I  G  F  G  S  F  V  D
451 CTGGGGGGTGACCTGCTCCGGGCCCTCAACGACATCACCGAGTCGGGCCGCATCGGTTTCGGGTCCTTCGTGGAC

176 K  T  V  L  P  F  V  N  T  H  P  E  K  L  R  N  P  C  P  N  K  E  K  Q  C
526 AAGACGGTGCTCCCTTTCGTCAACACGCACCCCGAGAAGCTGCGGAACCCCTGCCCCAACAAGGAGAAGCAGTGC

201 Q  P  P  F  A  F  R  H  V  L  K  L  T  D  N  S  K  Q  F  E  T  E  V  G  K
601 CAGCCCCCGTTCGCCTTCAGGCACGTGCTGAAGCTCACCGACAACTCCAAACAGTTCGAGACAGAAGTCGGGAAG

226 Q  L  I  S  G  N  L  D  A  P  E  G  G  L  D  A  M  M  Q  V  A  A  C  P  E
676 CAGCTGATCTCGGGGAACCTGGATGCCCCCGAGGGAGGGCTGGACGCCATGATGCAGGTGGCCGCGTGCCCGGAG

251 E  I  G  W  R  (N) V  T  R  L  L  V  F  A  T  D  D  G  F  H  F  A  G  D  G
751 GAAATCGGCTGGCGCAATGTCACCAGGTTGCTGGTGTTTGCCACGGATGATGGCTTCCACTTTGCGGGCGATGGA

276 K  L  G  A  I  L  T  P  N  D  G  R  C  H  L  E  D  N  L  Y  K  S  S  N  E
826 AAGCTGGGTGCCATCCTCACCCCCAACGACGGCCGCTGCCACCTGGAAGACAACCTGTACAAAAGCAGCAACGAA
```

```
 301 F  D  Y  P  S  V  G  Q  L  A  H  K  L  A  E  S  N  I  Q  P  I  F  A  V  T
 901 TTTGACTACCCATCGGTGGGCCAGCTGGCACACAAACTGGCAGAAAGCAACATCCAGCCCATCTTTGCGGTAACC

 326 K  K  M  V  K  T  Y  E  K  L  T  E  I  I  P  K  S  A  V  G  E  L  S  E  D
 976 AAGAAGATGGTGAAAACGTACGAGAAGCTGACGGAGATCATCCCCAAGTCTGCAGTCGGGGAGCTGTCTGAAGAT

 351 S  K  N  V  V  E  L  I  K  S  A  Y  N  K  L  S  S  R  V  F  L  D  H  N  T
1051 TCCAAGAACGTGGTGGAGCTTATCAAGAGTGCCTACAATAAACTGTCCTCCAGAGTCTTCCTGGATCACAACACC

 376 L  P  D  T  L  K  V  T  Y  D  S  F  C  S  K  G  V  S  K  V  D  Q  P  R  G
1126 CTCCCTGACACCCTGAAAGTCACCTACGACTCCTTCTGCAGTAAAGGGGTGTCGAAGGTGGACCAGCCCAGAGGG

 401 D  C  D  G  V  Q  I  N  V  P  I  T  F  Q  V  K  V  T  A  T  E  C  I  Q  E
1201 GACTGCGACGGCGTCCAGATCAACGTCCCGATCACCTTCCAGGTGAAGGTCACAGCCACCGAGTGCATCCAGGAA

 426 Q  S  F  T  I  R  A  L  G  F  T  D  T  V  T  V  R  V  L  P  Q  C  E  C  Q
1276 CAGTCCTTCACCATCCGGGCGCTGGGCTTTACGGACACGGTGACCGTGCGGGTCCTCCCCCAGTGCGAGTGCCAA

 451 C  R  D  A  S  R  D  R  S  V  C  G  G  R  G  S  M  E  C  G  V  C  R  C  D
1351 TGCCGGGACGCGAGCAGGGACCGCAGCGTCTGCGGTGGCAGAGGTTCGATGGAGTGCGGCGTCTGCAGGTGCGAC

 476 A  G  Y  I  G  K  N  C  E  C  Q  T  Q  G  R  S  S  Q  E  L  E  G  S  C  R
1426 GCCGGCTACATCGGGAAGAACTGCGAGTGCCAGACGCAGGGCCGGAGCAGCCAGGAGCTGGAGGGCAGCTGCCGC

 501 K  D  (N) S  S  I  I  C  S  G  L  G  D  C  I  C  G  Q  C  V  C  H  T  S  D
1501 AAGGACAACAGCTCCATCATCTGCTCGGGGCTGGGGGACTGCATCTGCGGGCAGTGCGTGTGCCACACGAGCGAC

 526 V  P  N  K  K  I  Y  G  Q  F  C  E  C  D  N  V  N  C  E  R  Y  D  G  Q  V
1576 GTGCCCAACAAGAAGATCTACGGCCAGTTCTGCGAGTGTGACAACGTCAACTGCGAACGCTACGACGGCCAAGTC

 551 C  G  G  D  K  R  G  L  C  F  C  G  T  C  R  C  Q  D  Q  Y  E  G  S  A  C
1651 TGCGGGGGCGACAAGAGGGGGCTCTGCTTCTGCGGCACCTGCAGGTGCCAGGACCAGTACGAGGGCTCGGCGTGC

 576 Q  C  L  K  S  T  Q  G  C  L  N  L  N  G  V  E  C  S  G  R  G  R  C  R  C
1726 CAGTGCCTCAAGTCCACGCAGGGCTGCCTCAACCTGAACGGCGTCGAGTGCAGCGGCCGCGGCCGGTGCCGCTGC

 601 N  V  C  Q  C  D  P  G  Y  Q  P  P  L  C  L  E  C  P  G  C  P  A  P  C  A
1801 AACGTGTGCCAGTGCGACCCCGGCTACCAGCCGCCCCTGTGCTTAGAGTGCCCCGGCTGCCCCGCACCCTGCGCC

 626 G  F  A  P  C  T  E  C  L  K  F  K  G  P  F  A  K (N) C  S  A  A  C  G  E
1876 GGCTTTGCCCCCTGCACCGAGTGCCTGAAGTTCAAGGGCCCCTTCGCCAAGAACTGCAGCGCAGCGTGCGGGGAG

 651 T  K  L  L  S  N  P  L  P  G  R  K  C  K  E  R  D  S  E  G  C  W  M  T  Y
1951 ACGAAGCTGCTGTCCAACCCGCTGCCCGGCCGCAAGTGCAAGGAGCGCGACTCGGAGGGCTGCTGGATGACCTAC

 676 T  L  V  Q  R  D  G  R  D  R  Y  D  V  H  V (N) D  T  R  E  C  V  K  G  P
2026 ACCCTGGTGCAGCGCGACGGGCGGGACAGATACGACGTGCACGTGAACGACACGCGCGAGTGTGTGAAGGGCCCC

 701 N  I  A  A  I  V  G  G  T  V  G  G  V  V  L  V  G  I  L  L  L  V  I  W  K
2101 AACATCGCGGCCATTGTGGGGGGCACCGTGGGGGGAGTTGTGCTGGTTGGCATCCTCCTGCTGGTCATCTGGAAG

 726 A  L  T  H  L  S  D  L  R  E  Y  H  R  F  E  K  E  K  L  K  S  Q  W  N  N
2176 GCCCTGACACACCTGAGCGACCTCAGGGAGTACCATCGCTTCGAGAAGGAGAAGCTCAAGTCCCAGTGGAACAAT

 751 D  N  P  L  F  K  S  A  T  T  T  V  M  N  P  K  F  A  E  S  *
2251 GATAACCCTCTTTTCAAGAGTGCCACCACGACAGTCATGAACCCTAAGTTTGCCGAGAGTTAGGGGTGCCTGGTG

2326 AAGACAAGGCCTTCTGCACCACCCAGATGGGACCACGCCCTCTCCACGTCCCCTCCAGCAGGCCGACCGTGACCC

2401 TGCTGCCTTGTGGACGTGGCTGATGATGCTTGACAACTCCACTGTTAACCAAAAATGCACTGCTTTTCCTGCCCC

2476 AGAATGATGGGCGTGACCAAATGATCCTATGGGCTCATGGTAAGGGCCAGCCTCCCCCTTGATGTCAATAACTTT
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2551 TGCTAGCAAGTCAGAGGAGGAATTGCCTACATTTTGTACGGTTACACACCAGTCCTTTGTAAAAATTAGCACAGC
2626 AGTCTGATGAAGAATTATTTATATGTGAACTTCTCAGGGTATGAAGTTATATCCCCTTGGTTATGCTGCCCCCCA
2701 ATCAATAAAAAAAATCAAGAAAAAAAAAAAAAAAA
```
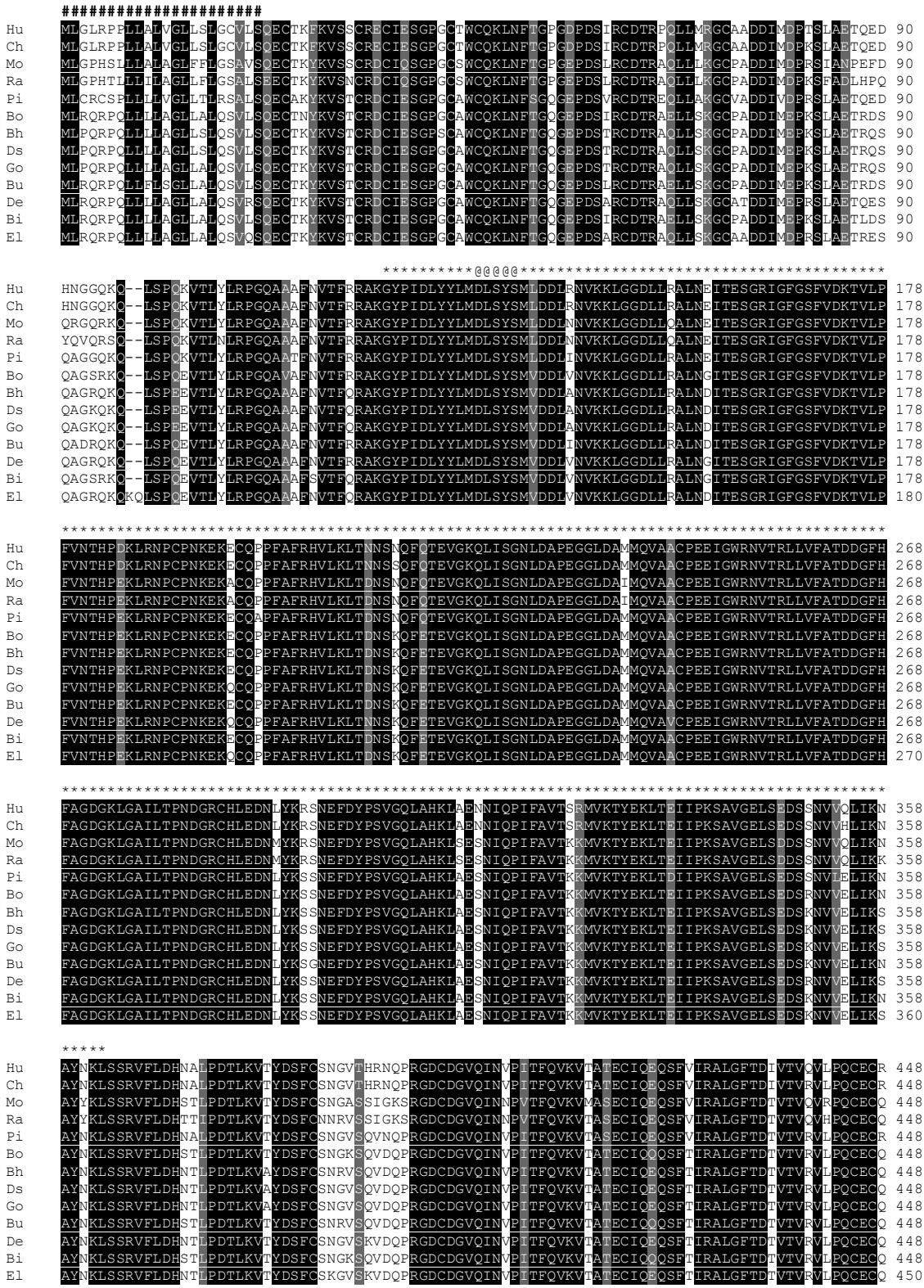
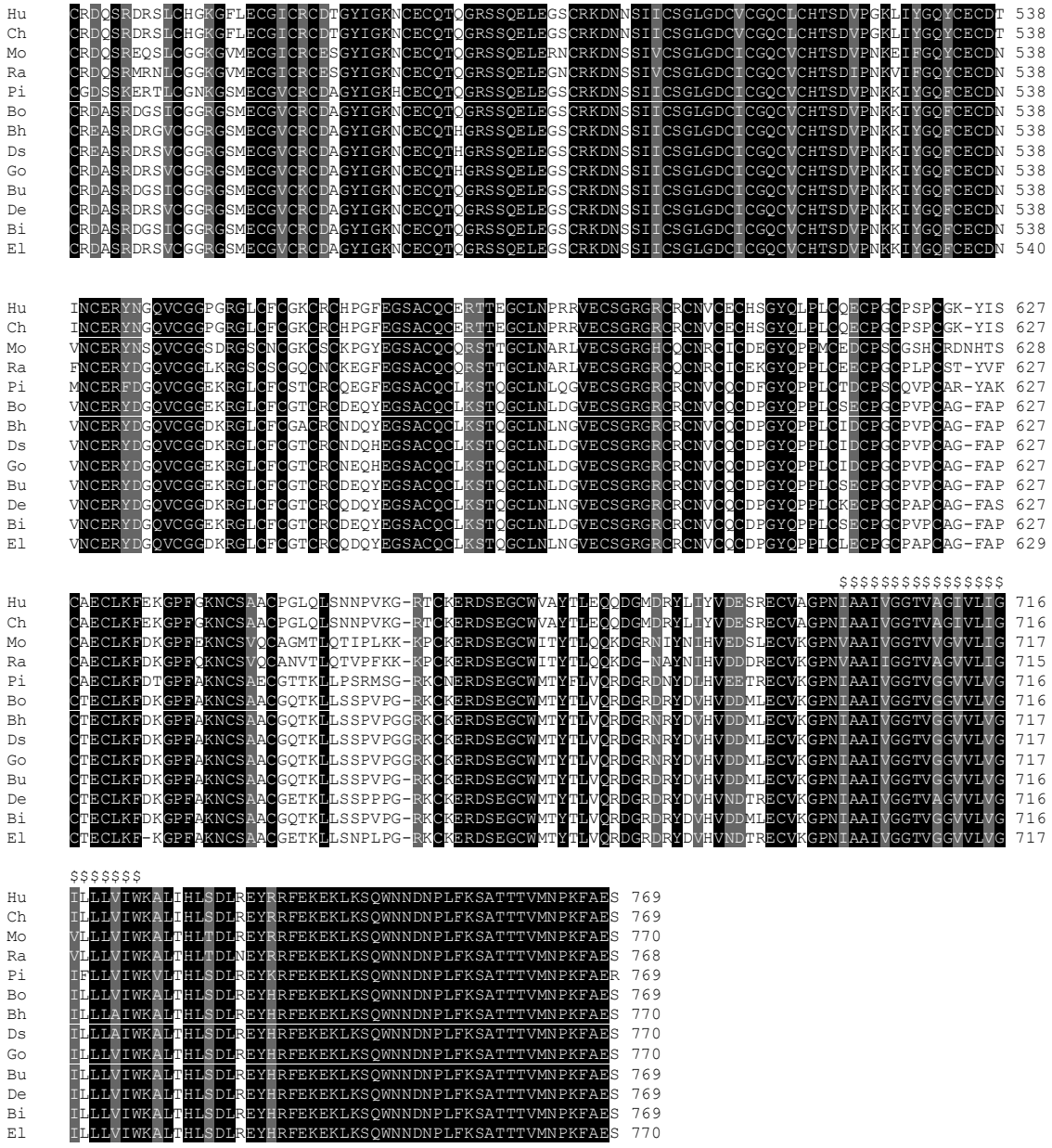

Fig. 2. The nucleotide and deduced amino acid sequence of the CD18 CDS of bison (A) deer (B) and elk (C). The predicted signal peptide sequences are marked with # on top of the amino acid residues. The putative transmembrane domain is underlined, and the *N*-glycosylation sites are circled.

Figure 3A

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Ch CRDQSRDRSLCHGKGFLECGICRCDTGYIGKNCECQTQGRSSQELEGSCRKDNNSIICSGLGDCVCGQCLCHTSDVPGKLIYGQYCECDT 538
Mo CRDQSREQSLCGGKGVMECGICRCESGYIGKNCECQTQGRSSQELERNCRKDNSSIVCSGLGDCICGQCVCHTSDVPNKEIFGQYCECDN 538
Ra CRDQSRMRNLCGGKGVMECGICRCESGYIGKNCECQTQGRSSQELEGNCRKDNSSIVCSGLGDCICGQCVCHTSDIPNKVIFGQYCECDN 538
Pi CGDSSKERTLCGNKGSMECGVCRCDAGYIGKHCECQTQGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
Bo CRDASRDGSLCGGRGSMECGVCRCDAGYIGKNCECQTQGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
Bh CREASRDRGVCGGRGSMECGVCRCDAGYIGKNCECQTHGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
Ds CREASRDRSVCGGRGSMECGVCRCDAGYIGKNCECQTHGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
Go CRDASRDRSVCGGRGSMECGVCRCDAGYIGKNCECQTHGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
Bu CRDASRDGSLCGGRGSMECGVCKCDAGYIGKNCECQTQGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
De CRDASRDRSVCGGRGSMECGVCRCDAGYIGKNCECQTQGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
Bi CRDASRDGSLCGGRGSMECGVCRCDAGYIGKNCECQTQGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 538
El CRDASRDRSVCGGRGSMECGVCRCDAGYIGKNCECQTQGRSSQELEGSCRKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDN 540
Hu INCERYNGQVCGGPGRGLCFCGKCRCHPGFEGSACQCERTTEGCLNPRRVECSGRGRCRCNVCECHSGYQLPLCQECPGCPSPCGK-YIS 627
Ch INCERYNGQVCGGPGRGLCFCGKCRCHPGFEGSACQCERTTEGCLNPRRVECSGRGRCRCNVCECHSGYQLPLCQECPGCPSPCGK-YIS 627
Mo VNCERYNSQVCGGSDRGSCNCGKCSCKPGYEGSACQCQRSTTGCLNARLVECSGRGHCQCNRCICDEGYQPPMCEDCPSCGSHCRDNHTS 628
Ra FNCERYDGQVCGGLKRGSCSCGQCNCKEGFEGSACQCQRSTTGCLNARLVECSGRGRCQCNRCICEKGYQPPLCEECPGCPLPCST-YVF 627
Pi MNCERFDGQVCGGEKRGLCFCSTCRCQEGFEGSACQCLKSTQGCLNLQGVECSGRGRCRCNVCQCDFGYQPPLCTDCPSCQVPCAR-YAK 627
Bo VNCERYDGQVCGGEKRGLCFCGTCRCDEQYEGSACQCLKSTQGCLNLDGVECSGRGRCRCNVCQCDPGYQPPLCSECPGCPVPCAG-FAP 627
Bh VNCERYDGQVCGGDKRGLCFCGACRCNDQYEGSACQCLKSTQGCLNLNGVECSGRGRCRCNVCQCDPGYQPPLCIDCPGCPVPCAG-FAP 627
Ds VNCERYDGQVCGGDKRGLCFCGTCRCNDQHEGSACQCLKSTQGCLNLDGVECSGRGRCRCNVCQCDPGYQPPLCIDCPGCPVPCAG-FAP 627
Go VNCERYDGQVCGGEKRGLCFCGTCRCNEQHEGSACQCLKSTQGCLNLDGVECSGRGRCRCNVCQCDPGYQPPLCIDCPGCPVPCAG-FAP 627
Bu VNCERYDGQVCGGEKRGLCFCGTCRCDEQYEGSACQCLKSTQGCLNLDGVECSGRGRCRCNVCQCDPGYQPPLCSECPGCPVPCAG-FAP 627
De VNCERYDGQVCGGDKRGLCFCGTCRCQDQYEGSACQCLKSTQGCLNLNGVECSGRGRCRCNVCQCDPGYQPPLCKECPGCPAPCAG-FAS 627
Bi VNCERYDGQVCGGEKRGLCFCGTCRCDEQYEGSACQCLKSTQGCLNLDGVECSGRGRCRCNVCQCDPGYQPPLCSECPGCPVPCAG-FAP 627
El VNCERYDGQVCGGDKRGLCFCGTCRCQDQYEGSACQCLKSTQGCLNLNGVECSGRGRCRCNVCQCDPGYQPPLCLECPGCPAPCAG-FAP 629
$$$$$$$$$$$$$$$$$
Hu CAECLKFEKGPFGKNCSAACPGLQLSNNPVKG-RTCKERDSEGCWVAYTLEQQDGMDRYLIYVDESRECVAGPNIAAIVGGTVAGIVLIG 716
Ch CAECLKFEKGPFGKNCSAACPGLQLSNNPVKG-RTCKERDSEGCWVAYTLEQQDGMDRYLIYVDESRECVAGPNIAAIVGGTVAGIVLIG 716
Mo CAECLKFDKGPFEKNCSVQCAGMTTQTIPLKK-KPCKERDSEGCWITYTLQQKDGRNIYNIHVEDSLECVKGPNVAAIVGGTVVGVVLIG 717
Ra CAECLKFDKGPFQKNCSVQCANVTTQTVPFKK-KPCKERDSEGCWITYTLQQKDG-NAAYNIHVDDDRECVKGPNVAAIIGGTVAGVVLIG 715
Pi CAECLKFDTGPFAKNCSAECGTTKLLPSRMSG-RKCNERDSEGCWMTYFLVQRDGRDNYDLHVEETRECVKGPNIAAIVGGTVGGVVLVG 716
Bo CTECLKFDKGPFAKNCSAACGQTKLLSSPVPG-RKCKERDSEGCWMTYTLVQRDGRDRYDVHVDDMLECVKGPNIAAIVGGTVGGVVLVG 716
Bh CTECLKFDKGPFAKNCSAACGQTKLLSSPVPGGRKCKERDSEGCWMTYTLVQRDGRNRYDVHVDDMLECVKGPNIAAIVGGTVGGVVLVG 717
Ds CTECLKFDKGPFAKNCSAACGQTKLLSSPVPGGRKCKERDSEGCWMTYTLVQRDGRNRYDVHVDDMLECVKGPNIAAIVGGTVGGVVLVG 717
Go CTECLKFDKGPFAKNCSAACGQTKLLSSPVPGGRKCKERDSEGCWMTYTLVQRDGRNRYDVHVDDMLECVKGPNIAAIVGGTVGGVVLVG 717
Bu CTECLKFDKGPFAKNCSAACGQTKLLSSPVPG-RKCKERDSEGCWMTYTLVQRDGRDRYDVHVDDMLECVKGPNIAAIVGGTVAGVVLVG 716
De CTECLKFDKGPFAKNCSAACGETKLLSSPPPG-RKCKERDSEGCWMTYTLVQRDGRDRYDVHVNDTRECVKGPNIAAIVGGTVAGVVLVG 716
Bi CTECLKFDKGPFAKNCSAACGQTKLLSSPVPG-RKCKERDSEGCWMTYTLVQRDGRDRYDVHVDDMLECVKGPNIAAIVGGTVGGVVLVG 716
El CTECLKF-KGPFAKNCSAACGETKLLSNPLPG-RKCKERDSEGCWMTYTLVQRDGRDRYDVHVNDTRECVKGPNIAAIVGGTVGGVVLVG 717
$$$$$$$
Hu ILLLVIWKALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 769
Ch ILLLVIWKALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 769
Mo VLLLVIWKALTHLTDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 770
Ra VLLLVIWKALTHLIDLNEYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 768
Pi IFLLVIWKVLTHLSDLREYKRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAER 769
Bo ILLLVIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 769
Bh ILLLAIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 770
Ds ILLLAIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 770
Go ILLLVIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 770
Bu ILLLVIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 769
De ILLLVIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 769
Bi ILLLVIWKALTHLSDLREYHRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 769
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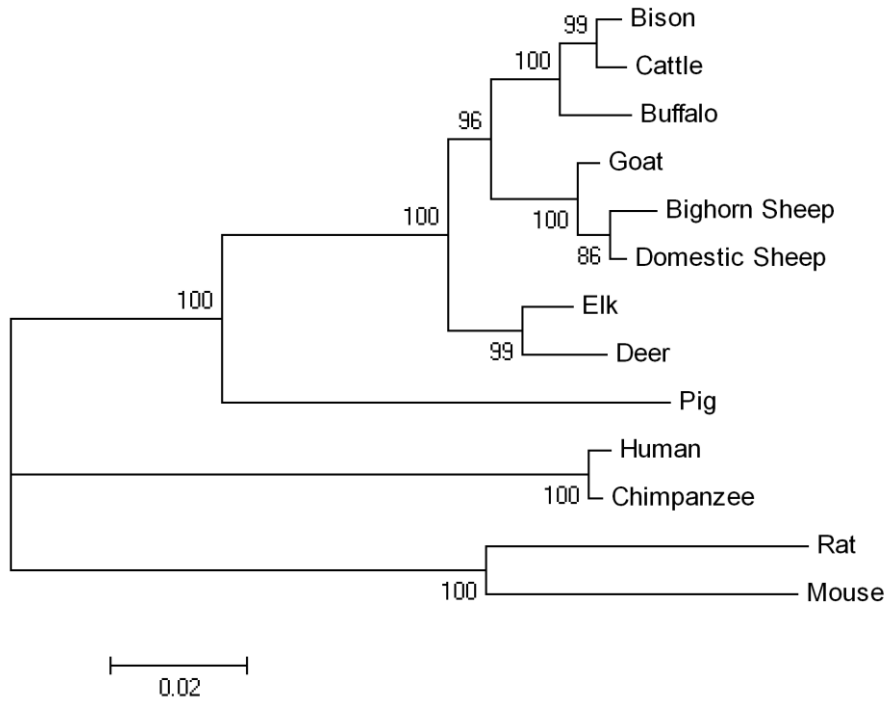
Figure 3B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Bison
100
Cattle
Buffalo
96
Goat
100
100
Bighorn Sheep
86
Domestic Sheep
100
Elk
99
Deer
Pig
Human
100
Chimpanzee
Rat
100
Mouse
0.02

Fig. 3. (A) Comparison of the deduced amino acid sequences of CDS of bison (Bi), deer (De), and elk (El) CD18 with that of human (Hu), chimpanzee (Ch), mouse (Mo), rat (Ra), pig (Pi), cattle (Bo), bighorn sheep (Bh), domestic sheep (Ds), goat (Go) and buffalo (Bu). Identical residues are indicated by white text on black. The conserved domains and motifs are marked on top of the residues: putative signal peptide (#), I-like domain (*), MIDAS motif (@), and membrane spanning domain ($). (B) Phylogenetic analysis of CD18 of bison, deer and elk and other species. Phylogenetic tree was constructed by neighbor-joining using mega 4.

CHAPTER TWO

The intact signal peptide of CD18, the beta subunit of beta2-integrins, renders ruminants susceptible to *Mannheimia haemolytica* leukotoxin

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ABSTRACT

Signal peptides of membrane proteins are cleaved by endoplasmic reticulum-resident signal peptidase, and hence are not present on mature membrane proteins. Here we report that, contrary to the paradigm, the signal peptide of ruminant CD18, the β subunit of β_2-integrins, is not cleaved. Intriguingly, the intact signal peptide of CD18 is responsible for the susceptibility of ruminant leukocytes to *Mannheimia (Pasteurella) haemolytica* leukotoxin. Inhibition of leukotoxin-induced cytolysis of ruminant leukocytes by CD18 peptide analogs revealed that the leukotoxin-binding site is formed by amino acids 5-17 of CD18 which, surprisingly, comprise most of the signal sequence. Flow cytometric analysis of ruminant leukocytes indicated the presence of the signal peptide on mature CD18 molecules expressed on the cell surface. Analysis of transfectants expressing CD18 containing the 'FLAG' epitope at the putative cleavage site confirmed that the signal peptide of bovine CD18 is not cleaved. Analysis of the signal sequence of CD18 of eight ruminants and five non-ruminants revealed that the signal sequence of CD18 of ruminants contains 'cleavage-inhibiting' glutamine (Q), whereas that of non-ruminants contains 'cleavage-conducive' glycine (G) at position -5 relative to the cleavage site. Site-directed mutagenesis of Q to G at position -5 of the signal peptide of bovine CD18 resulted in the cleavage of the signal peptide and abrogation of cytolysis of transfectants expressing bovine CD18 carrying the Q(-5)G mutation. We propose that engineering cattle and other ruminants to contain this mutation would provide a novel technology to render them less susceptible to pneumonic pasteurellosis and concomitant economic losses.

INTRODUCTION

The nascent membrane protein contains a signal sequence that directs the protein/ribosome to the endoplasmic reticulum (ER) membrane (1-3). The signal peptide binds to the signal recognition particle (SRP) which in turn binds to the SRP receptor on the ER membrane and helps in the translocation of the protein into the lumen of the ER. The signal peptide is cleaved from the protein by the ER-resident signal peptidase while it is still growing on the ribosome. Thus the signal peptide is not present on the mature protein that reaches the plasma membrane following post-translational modifications. Our studies aimed at mapping the *Mannheimia (Pasteurella) haemolytica* leukotoxin (Lkt) binding site on its receptor CD18 have led to the unexpected finding that the signal peptide of ruminant CD18 remains intact on the mature CD18 molecule on the leukocytes of ruminants, and renders these cells susceptible to cytolysis by Lkt.

M. haemolytica is the most important bacterial pathogen of respiratory disease in cattle, and other domestic and wild ruminants (4-7). This disease, commonly known as pneumonic pasteurellosis or shipping fever in cattle, has been estimated to cost over $1 billion to the cattle industry of US alone (8). *M. haemolytica* is a gram negative coccobacillus commonly found as a commensal in the tonsillar crypts and upper respiratory tract of healthy ruminants (9). In conjunction with active viral infection and stress factors, the organism multiplies rapidly, reaches the lungs, and causes an acute fibrinonecrotic pleuropneumonia (10).

This organism produces several virulence factors which include the capsule, outer membrane proteins, adhesins, neuraminidase, lipopolysaccharide and leukotoxin (Lkt; 11). Based on the observation that Lkt-deletion mutants of *M. haemolytica* cause reduced mortality and much milder lung lesions than the wild-type organisms, Lkt is accepted as the most important virulence factor of this organism (12-15). *M. haemolytica* Lkt belongs to the family of Gram-negative

bacterial exotoxins, referred to as RTX (Repeats in toxins) toxins. It shares extensive homology with the exotoxins produced by *Escherichia coli* (16), *Actinobacillus pleuropneumoniae* (17), and *Actinobacillus actinomycetemcomitans* (18). However, cytolytic activity of *M. haemolytica* Lkt is specific for ruminant leukocytes (19, 20). Although all subsets of leukocytes are susceptible to the cytolytic effects of Lkt, polymorphonuclear leukocytes (PMNs) are the most susceptible subset (21). PMN-depletion mitigates the lung injury in calves caused by *M. haemolytica* infection (22). Therefore, Lkt-induced PMN lysis and degranulation are the primary causes of acute inflammation and lung injury characteristic of pneumonic pasteurellosis (22-24).

Previously, we and others identified β_2-integrins as the receptor for Lkt on bovine leukocytes (25-28). β_2-integrins are leukocyte-specific integrins which mediate several functions of leukocytes including homing into areas of inflammation, phagocytosis, antigen presentation and cytotoxicity. They have a common β subunit, CD18, which associates with four α subunits resulting in four different integrins CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (CR4) and CD11d/CD18 (29, 30). We have previously demonstrated that CD18 is necessary and sufficient to mediate Lkt-induced cytolysis of bovine and ovine leukocytes (31-34), and mapped the Lkt binding site on bovine CD18 to lie between amino acids (aa) 1-291 (35). The next logical step was to identify the precise binding site of Lkt on CD18 which formed the objective of this study. Here we report that the Lkt-binding site is formed by aa 5-17 of CD18 which, surprisingly, comprise most of the aa of the signal peptide which remains intact on mature CD18 molecules on the cell surface. More importantly, a single aa substitution in CD18 results in the cleavage of the signal peptide which abrogates Lkt-induced cytolysis of the target cells.

MATERIALS AND METHODS

Cell lines and antibodies. The cell lines BL-3 (bovine lymphoma), and P815 (murine mastocytoma) were propagated in complete Dulbecco's minimum Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 ug/ml of gentamicin (complete medium). The transfectant 2B2, expressing full-length bovine CD18 on the cell surface, was previously developed in our laboratory by transfecting P815 with cDNA for bovine CD18 (31). The transfectants, BFL and BQG were selected and propagated in the complete DMEM together with 500 ug/ml of Geneticin (G418, Invitrogen). PMNs were isolated from peripheral blood by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech.), followed by hypotonic lysis of the erythrocyte pellet, as previously described (26). Anti-bovine CD18 MAb, BAQ30A, was obtained from The Washington State University Monoclonal Antibody Center. MAbs M1 and M2 specific for the 'FLAG' epitope were obtained from Sigma.

Peptides. A nested set of 20-mer peptides spanning aa 1-291 and aa 500-600 of bovine CD18 was synthesized at Sigma Genosys. Once the peptide which inhibits Lkt-induced cytotoxicity was identified, another set of peptides was synthesized with N-terminal truncation by dropping one aa at a time while keeping the C-terminal aa constant. Once the N-terminal aa of the minimal peptide was identified, another set of peptides was synthesized with C-terminal truncation by dropping one aa at a time while keeping the N-terminal aa constant. All the peptides were resuspended in dimethysulfoxide (ATCC) at a concentration of 10 mg/ml, aliquoted and stored at -20^{o}C.

Preparation of Lkt. Production of Lkt from *M. haemolytica* A1 has been described by us previously (48). Briefly, *M. haemolytica* was grown to logarithmic phase in brain heart infusion

broth (Remel, Lenexa, KS) at 37^0C, collected by centrifugation (13,500 X g for 20 min at 4^0C), and resuspended in twice the original culture volume of colorless RPMI 1640 medium. After an additional 1-1.5 hours of growth at 37^0C, the bacteria were removed from the culture by centrifugation (13, 500 X g for 20 min at 4^0C). Culture supernatant was filter-sterilized and crude toxin preparations were aliquoted and stored at -20^0C until needed. The undiluted toxin preparation contained 640 Units of toxin per ml. All experiments were performed with the same batch of toxin.

Detection of inhibition of Lkt-induced cytolysis of target cells by the peptide analogs of CD18. The MTT [3-(4,5-dimethylthiazoyl-2-YI]-2,5-diphenyl tetrazolium bromide; Sigma] dye reduction cytotoxicity assay for detection of Lkt-induced cytolysis of target cells has been previously described by us (48). This assay measures the ability of the ER-resident enzymes in viable cells to convert a tetrazolium dye into a purple formazan precipitate, which is later dissolved in acid isopropanol. The optical density (OD) of the end product, representing the intensity of the purple color developed, is directly proportional to the viability of the cells. Briefly, the target cells were resuspended in colorless RPMI 1640 (without neutral red) at a concentration of 5 X 10^6 cells ml^{-1}, and seeded into 96 well round bottom microtiter plates (50 ul/well) containing the serially diluted Lkt in duplicates and incubated at 37^0C for 1 hour. Cells were centrifuged at 600 X g for 5 min following incubation, and the supernatant fluid was discarded. The cell pellets were resuspended in 100 ul of colorless RPMI 1640 and 20 ul of 0.5% MTT dye were added to each well. After 30 min of incubation at 37^0C, the plates were centrifuged at 600 X g for 5 min and the supernatant fluid was removed. The formazan precipitate was thoroughly dissolved in 100 ul acid isopropanol and the OD of the samples was measured using an ELISA

reader at 540 nm. The percent cytotoxicity was calculated as follows: % cytotoxicity = [1-(OD of toxin-treated cells/OD of toxin-untreated cells)] X 100. Inhibition of Lkt-induced cytolysis of target cells by the peptide analogs was detected by the MTT assay with the obvious exception that Lkt was pre-incubated with the appropriate concentration of 50 ul peptides before incubation with the target cells. Lkt was used at a concentration (20 Units ml^{-1}) that causes 50% cytolysis of target cells. The percent inhibition of cytolysis was calculated as follows: % Inhibition of cytolysis = [1 – (% cytolysis in the presence of peptide/ % cytolysis in the absence of peptide)] x 100.

Cloning and expression of bovine CD18 carrying the 'FLAG' epitope at the cleavage site. The *GeneTailor site-directed mutagenesis system* (Invitrogen) was used to insert the 'FLAG' epitope (DYKDDDDK) into the vector pMD1 carrying bovine CD18 cDNA, at the signal peptide cleavage site (between aa 22 and 23). The insertion was carried out in two steps (12bp at a time). The insertion of 'FLAG' epitope into CD18 was confirmed by DNA sequencing. The vector carrying the 'FLAG'-tagged CD18 was transfected into P815 cells with Lipofectamine^{TM} 2000 according to the manufacturer's protocol.

Cloning and expression of bovine CD18 carrying the Q(-5)G mutation. The bovine cDNA for CD18 was previously subcloned into the mammalian expression vector pCI-neo to yield pMD1 (31). To produce Q(-5)G mutation in bovine CD18, site-directed mutagenesis was performed using the *GeneTailor site-directed mutagenesis* system (Invitrogen). The CD18 sequence after the point mutation was checked by DNA sequencing. Transfection of P815 cells with Lipofectamine^{TM} 2000 (Invitrogen) was carried out according to the manufacturer's recommendations.

Flow cytometric analysis. Transfectants, 2B2, BFL and BQG were examined for the cell surface expression of bovine CD18 using anti-CD18 MAb, BAQ30A, by flow cytometry, as previously described by us (31). Briefly, 5 X 10^6 cells ml^{-1} in 50 ul of FACS buffer (3% horse serum and 0.01% sodium azide in PBS), were incubated with 50 ul of MAb, BAQ30A, (15 ug/ml) at 4^0C for 20 min. Following three washes in FACS buffer, the cells were incubated with 50 ul of FITC-conjugated goat anti-murine Ig Ab (Caltech Laboratories, Burlington, CA; 1:200 dilution) at 4^0C for 20min. The parent cells (P815) used as the negative controls were treated similarly. The cells were washed three times with FACS buffer, resuspended in PBS and analyzed by a flow cytometer (FACSort, Becton-Dickinson Immunocytometry Systems., San Jose, CA). PMNs of ruminants (cattle, goats, domestic sheep, wild sheep, deer and bison), and the Jurkat and RAW264.1 cells were examined for the presence of intact signal peptide of CD18 using the chicken antiserum developed against the synthetic peptide spanning aa 5-19 of bovine CD18 signal peptide (Sigma Genosys), as described above. Transfectants, 2B2 and BFL were tested for the cell surface expression of FLAG epitope using MAbs, M1 and M2, by flow cytometry, as described above.

Western Blot Analysis. Cell lysates of 2B2 and BQG were prepared as described by us earlier (26). Briefly, 1 X 10^8 cells were suspended in 1 ml of ice-cold lysis buffer (0.5% NP-40, 5 mM MgCl2, 50 mM Tris, pH 7.5) containing 10 ul/ml of cocktail protease inhibitor (Promega) and incubated on ice for 40 min. The lysate was centrifuged (14, 000 X g) for 10 min at 4^0C and supernatant fluid was aliquoted and stored at -20^0C. Cell lysates were subjected to SDS-PAGE followed by western blot analysis with the anti-bovine CD18 MAb BAQ30A. Horseradish

peroxidase-conjugated goat anti-mouse Ig Abs were used as the secondary antibody (Calbiochem). The blots were developed by using chemiluminescence detection system (Bio-rad).

Statistical Analysis

One-way ANOVA was employed to determine whether the differences in % inhibition caused by the different peptides are statistically significant.

RESULTS

Peptide analogs of bovine CD18 inhibit Lkt-induced cytolysis of target cells.

Binding of a ligand to its receptor can be inhibited by synthetic peptides representing the aa involved in the binding (36, 37). In this study, we employed bovine CD18 peptide analogs to identify the binding site of *M. haemolytica* Lkt on bovine CD18. Inhibition of Lkt-induced cytolysis of target cells by a nested set of 20-mer peptides spanning aa 1-291 of bovine CD18 was used for this purpose. Since the most pronounced difference in the aa sequence of CD18 of ruminants and non-ruminants was observed in the N-terminal region (aa 1-29), the point of origin of the first 5 peptides was staggered by 5 aa while the rest of the peptides were staggered by 15 aa. All the peptides were tested for their ability to inhibit Lkt-induced cytolysis of target cells by the MTT dye-reduction cytotoxicity assay. As target cells, we used BL-3 cells (bovine lymphoma cell-line) in these experiments since they are readily available. The findings with BL-3 cells were subsequently confirmed with PMNs of cattle and other ruminants. Two peptides, P1 and P5 strongly inhibited Lkt-induced cytolysis of BL-3 cells (Fig. 1A). Comparison of the concentration of P1 and P5 which causes 50% inhibition of Lkt-induced cytolysis of BL-3 cells revealed the

potency of P5 to be higher than that of P1 (1.7 ug/ml versus 17 ug/ml; Fig. 1B). Two other peptides containing the same aa as P5, but in a randomly scrambled sequence, failed to inhibit Lkt-induced cytolysis of BL-3 cells indicating that the inhibition of Lkt-induced cytolysis by P5 was specific (Fig. 1C).

CD18 peptide analog consisting of aa 5-17 is the minimal peptide that inhibits Lkt-induced cytolysis of target cells.

In order to identify the minimal peptide that would inhibit Lkt-induced cytolysis of target cells as efficiently as P5, peptides with N- and C- terminal truncations were used in the cytotoxicity assay. The % inhibition given by peptide P5 was significantly higher than that given by the other peptides with N-terminal truncation indicating that the aa #5 is the N-terminal aa of the minimal peptide (Fig. 2A). Subsequently, another set of peptides were synthesized with C-terminal truncation by dropping one aa at a time while keeping the N-terminus constant at aa #5. When the aa from the C-terminus dropped to the 16^{th}, the % inhibition decreased indicating that the aa #17 is the C-terminal aa of the minimal peptide (Fig. 2B). Hence the peptide composed of aa 5-17 is the minimal peptide analog of bovine CD18 that effectively inhibits Lkt-induced cytolysis of BL-3 cells. The peptide P17 (aa 5-17) effectively inhibited the Lkt-induced cytolysis of PMNs of cattle, goats, domestic sheep, wild sheep, deer, and bison, as well (Fig. 3), confirming the finding with BL-3 cells that aa 5-17 is the minimal peptide analog of bovine CD18 that effectively inhibits Lkt-induced cytolysis of target cells. These results indicated that the domain encompassed by aa 5-17 of CD18 is the minimal aa sequence of CD18 that could serve as the receptor for Lkt on ruminant leukocytes.

The aa sequence of bovine CD18 peptide analog that inhibits Lkt-induced cytolysis is from the signal sequence of CD18

The aa 5-17 constitute 13 out of the 22 aa of the predicted signal sequence of CD18. Therefore the next logical step was to determine whether the signal peptide remains intact on the mature cell surface CD18 of ruminant leukocytes. Flow cytometric analysis with a chicken antiserum against bovine CD18 signal peptide revealed that the signal peptide indeed remains intact on the CD18 molecules on the PMNs of ruminants (cattle, goats, domestic sheep, wild sheep, deer, and bison; Fig. 4). The mouse macrophage cell-line RAW264.A and the human T cell leukemia cell-line Jurkat were not stained by the anti-signal peptide serum indicating the absence of signal peptide on the CD18 of these cells of non-ruminant origin.

The signal peptide of ruminant CD18 is not cleaved from the mature protein

In order to confirm the fact that the signal peptide of bovine CD18 is not cleaved, the minigene encoding the 'FLAG' epitope (DYKDDDDK; 38) was introduced at the putative signal peptide cleavage site (between aa 22 and 23). 'FLAG'-tagged CD18 was transfected into the murine mastocytoma cell-line, P815, which is non-susceptible to *M. haemolytica* Lkt-induced cytolysis. A transfectant, designated BFL, stably expressing bovine CD18 containing the 'FLAG' epitope between aa 22 and 23 was tested with two monoclonal antibodies (MAbs), M1 and M2, specific for the 'FLAG' epitope. M1 recognizes the free N-terminal end of 'FLAG', while M2 recognizes 'FLAG' irrespective of its sequence context (Fig. 5A; 38). The MAb M2 bound to the transfectant carrying 'FLAG'-tagged CD18 (BFL) confirming the expression of 'FLAG'-epitope on the cell surface (Fig. 5B). The MAb M1 did not bind to the transfectants expressing 'FLAG'-bearing CD18, indicating the lack of cleavage of the signal peptide on the mature membrane CD18

(Fig. 5C). The lack of binding of MAbs M1 and M2 to the transfectant 2B2 (expressing bovine CD18 without the 'FLAG'-epitope, 31) indicated the specificity of these two MAbs for the 'FLAG' epitope (Fig. 5B and 5C). The level of expression of CD18 on the transfectant BFL was comparable to that of the transfectant 2B2 confirming that the lack of binding of M1 to BFL was not due to low expression of CD18 on BFL (Fig. 5D).

The signal peptide of CD18 of ruminants contains 'cleavage-inhibiting' glutamine at position -5 relative to the cleavage site.

Our finding that the aa 5-17 within the signal peptide of ruminant CD18 serves as the receptor for *M. haemolytica* Lkt, and the fact that the cytolytic activity of Lkt is absolutely specific for ruminant leukocytes, prompted us to examine the signal sequences of CD18 of ruminants (n=8) and non-ruminants (n=5) (Fig. 6A). The predicted signal sequence of both the ruminant and non-ruminant CD18 contains 22 aa. The "-3 -1 rule" of Von Hejne (39) for signal peptide cleavage calls for the presence of aa with small uncharged side chains at position -1 and -3 relative to the cleavage site (Fig. 6B). Both ruminant and non-ruminant CD18 signal peptides conform to this rule. The aa residue at position -5 could also determine whether the signal peptide gets cleaved or not (40). Helix-breaking residues glycine and proline are conducive for signal peptide cleavage (39). Arginine is also conducive to signal peptide cleavage (39, 40). Glutamine on the other hand has been shown to inhibit cleavage of the signal peptide (40). Surprisingly, CD18 of all eight ruminants examined contain 'cleavage-inhibiting' glutamine, while CD18 of all five non-ruminants examined contain the 'cleavage-conducive' glycine (humans, mice, rats, and chimpanzees) or arginine (pigs; Fig. 6A).

Mutation of Q to G at position -5 relative to the cleavage site of CD18 signal peptide abrogates Lkt-induced cytolysis.

The observation that the signal peptide of CD18 of ruminants (Lkt-susceptible) contains Q at -5 position whereas that of non-ruminants (Lkt-non-susceptible) contains G prompted us to ask whether site-directed mutagenesis of Q to G [Q(-5)G] would result in the abrogation of Lkt-induced cytolysis of transfectants expressing Q(-5)G mutation in the signal peptide of CD18. The aa Q at -5 position of bovine CD18 was mutated to G, and the mutated CD18 was transfected into P815 cell-line which is non-susceptible to Lkt-induced cytolysis. A transfectant stably expressing bovine CD18 carrying the Q(-5)G mutation, designated as BQG, was selected for further analysis. Flow cytometric analysis with an anti-CD18 MAb revealed that the surface expression of bovine CD18 by this transfectant BQG was comparable to that of the transfectant, 2B2, expressing wild type CD18 (Fig. 7A). Interestingly, the transfectant 2B2 was effectively lysed by Lkt in a concentration-dependent manner, whereas the transfectant BQG was not lysed at all, indicating that the Q(-5)G mutation in CD18, in deed, abrogates Lkt-induced cytolysis of target cells (Fig. 7B).

Abrogation of Lkt-induced cytolysis of transfectants expressing bovine CD18 carrying the Q(-5)G mutation is due to the cleavage of the signal peptide.

The abrogation of cytolysis of the transfectant BQG could be due to (1) the difference in structural features of glutamine and glycine or (2) the cleavage of the signal peptide. To elucidate the mechanism underlying the abrogation of cytolysis, western blot analysis of cell lysates of BQG and 2B2 was performed with the anti-CD18 MAb. Single bands representing CD18 were detected with both cell lysates around 95kDa. However, the CD18 from BQG exhibited a lower molecular

weight than the CD18 from 2B2 (Fig. 7C) suggesting that the replacement of cleavage-inhibiting glutamine by the cleavage-conducive glycine at -5 position of bovine CD18 resulted in the cleavage of signal peptide.

DISCUSSION

Previously, we have mapped the Lkt-binding site to lie betweem aa 1-291 of bovine CD18. In this study, by employing synthetic peptides spanning aa 1-291 in inhibition of Lkt-induced cytolysis assays, we have shown that the CD18 domain formed by aa 5-17 represents the Lkt-binding site on CD18 of bovine PMNs (Fig. 3). Furthermore, inhibition of Lkt-induced cytolysis of PMNs of goats, domestic sheep, wild sheep, deer and bison by peptide P17 (aa 5-17) indicates that the CD18 domain formed by aa 5-17 represents the Lkt-binding site on CD18 of other ruminants as well.

Our finding that the aa 5-17 of bovine CD18 serves as the binding site for Lkt is not in agreement with the finding of Dileepan *et al.* (41, 42). These workers have previously reported that the Lkt binding site lies within aa 500-600 of bovine CD18, more specifically, between aa 541-581. Our results clearly indicate that this conclusion is incorrect for the following reasons: **1.** Two different sets of synthetic peptides spanning aa 500-600 failed to inhibit Lkt-induced cytolysis of bovine PMNs (Fig. 8); **2.** Synthetic peptides containing the signal sequence aa 5-17 effectively inhibited Lkt-induced cytolysis of PMNs of cattle and other ruminants (Fig. 3). If Lkt binds to CD18 between aa 541 and 581, one would expect to see cytolysis of target cells when Lkt is incubated with a synthetic peptide containing aa 5-17 only. But the target cells were not lysed (Fig. 1 & 2); **3.** Our transfectants expressing CD18 containing the Q(-5)G mutation in the signal peptide are not lysed by Lkt (Fig. 7) although the aa 500 to 600 are intact in the CD18. The failure

of Dileepan *et al* (41, 42) to identify aa 5-17 in the signal peptide as the Lkt binding site is very likely due to the fact that their transductants were developed with K562 cells. K562 cells transfected with bovine CD18 do not express CD18 with intact signal peptide (unpublished observations). Therefore, it is very likely that K562 cells carry a signal peptidase that cleaves the signal peptide in spite of the presence of Q at position -5. We also note that K562 is a poorly characterized cell-line and the literature (43, 44) contains conflicting reports as to its lineage (erythroleukemia and granulocyte). Our studies indicate that the findings of Dileepan *et al*. (41, 42) are unique to bovine CD18 transductants developed with K562 cells, and do not reflect the molecular events occurring in ruminant leukocytes.

Our finding that the CD18 domain formed by aa 5-17 is the Lkt-binding site on CD18 revealed interesting information on the signal peptide of CD18 of ruminants. The aa 5-17 comprise 13 out of 22 aa of the predicted sequence of bovine CD18 signal peptide, which suggests that the signal peptide of cattle, and other ruminants, may not be cleaved. Paradigm dictates that signal peptides of plasma membrane proteins are cleaved by the signal peptidase in the ER (1-3). However, a few membrane proteins present an exception to this paradigm. Corticotrophin releasing factor receptor type 2a (45), prion protein (46), and sucrase isomaltase (type II; 47) represent the very small minority of proteins with intact signal peptide on the mature molecule.

Flow cytometric analysis with the anti-bovine CD18 signal peptide serum supported our view that the signal peptide of the CD18 of ruminants remains intact on the mature cell-surface CD18 (Fig. 4). The introduction of the FLAG epitope at the cleavage site and the analysis of transfectants expressing FLAG-tagged CD18 confirmed that the signal peptide of CD18 of cattle is indeed not cleaved from the mature CD18 molecule (Fig. 5). It is very likely that the signal peptide of CD18 of other ruminants is not cleaved as well since $\geq$95% aa sequence identity exist

among them. Reactivity of PMNs from all the ruminants with the chicken antiserum against bovine signal peptide strongly supports this notion.

The presence of cleavage-inhibiting Q, instead of the cleavage-conducive G, at position -5 relative to the cleavage site of CD18 of all ruminants is very likely responsible for the failure of the ruminant signal peptide to be cleaved (Fig. 7). This view is strongly supported by the fact that the Q(-5)G mutation in the signal peptide of bovine CD18 results in the cleavage of signal peptide as revealed by the western blot analysis (Fig. 7C), and the resultant abrogation of Lkt-induced cytolysis of the transfectants (Fig. 7B). It is really interesting that a single aa substitution in bovine CD18 eliminates the susceptibility of transfectants expressing CD18 carrying the substitution. This finding is of great practical significance. Leukotoxin is the most important virulence factor of *M. haemolytica* (12, 13, 23, 24). Leukotoxin deletion mutants of *M. haemolytica* induce milder lung lesions and reduced mortality (12-15). Therefore, cattle and other ruminants that express CD18 without the signal peptide on their leukocytes could be expected to be much less susceptible to *M. haemolytica*-caused pneumonia. Our finding that the Q(-5)G mutation in the signal peptide of bovine CD18 results in the cleavage of the signal peptide and abrogation of Lkt-induced cytolysis of the transfectants paves the way for the genetic engineering of cattle and other ruminants that are less susceptible to *M. haemolytica*-caused pneumonia.

In summary, we have demonstrated that the aa 5-17 within the signal peptide of ruminant CD18 serves as the receptor for *M. haemolytica* Lkt, and that the failure of the signal peptide to be cleaved from mature CD18 molecules renders the ruminant leukocytes susceptible to Lkt. We propose that engineering cattle and other ruminants to contain this mutation would provide a novel technology to render them less susceptible to pneumonic pasteurellosis and concomitant economic losses.

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Figure 1.

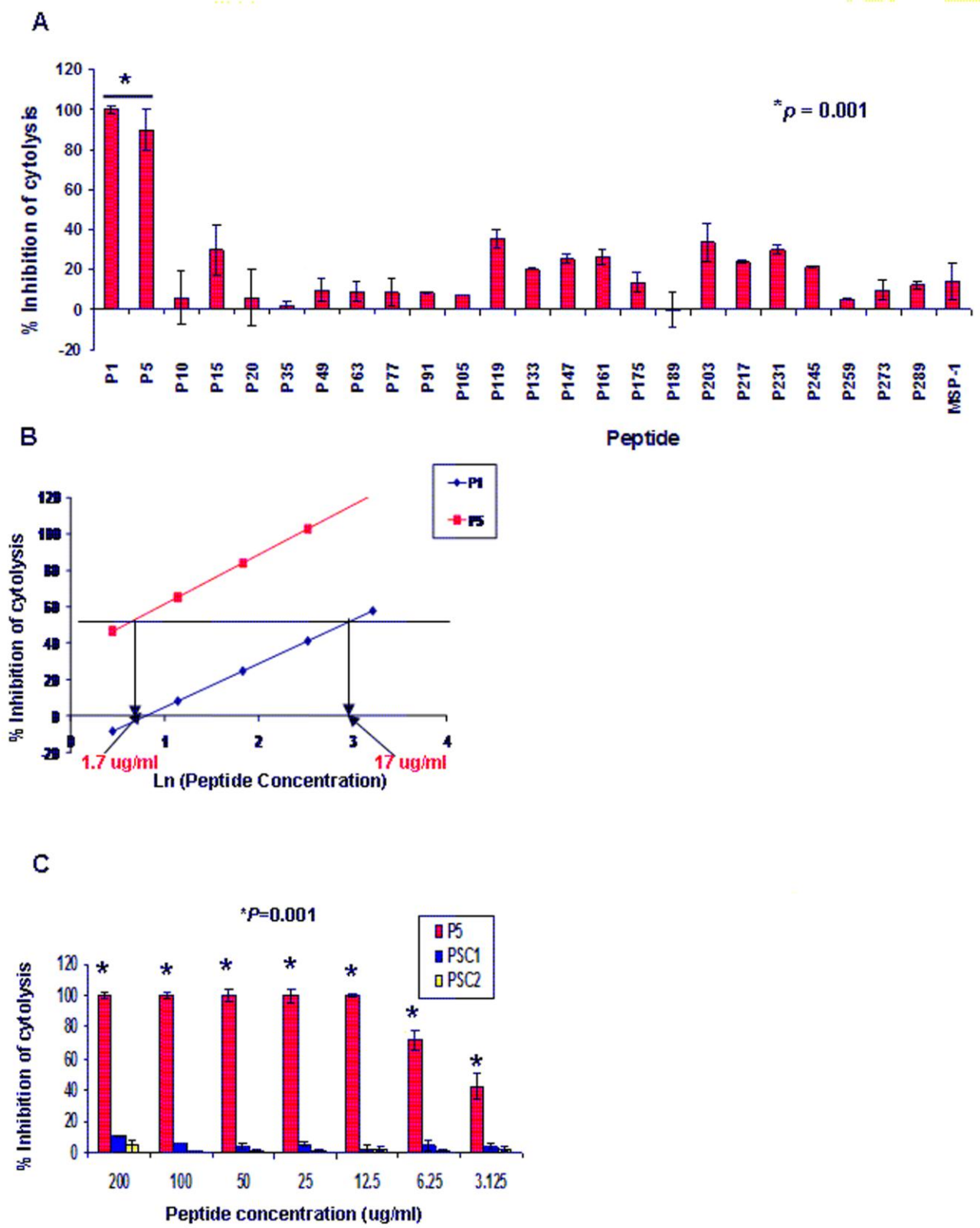
A
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
*
*p = 0.001
P1
P5
P10
P15
P20
P35
P49
P63
P77
P91
P105
P119
P133
P147
P161
P175
P189
P203
P217
P231
P245
P259
P273
P289
MSP-1
Peptide
B
% Inhibition of cytolysis
P1
P5
1.7 ug/ml
17 ug/ml
Ln (Peptide Concentration)
C
*P=0.001
P5
PSC1
PSC2
% Inhibition of cytolysis
120
100
80
60
40
20
0
200
100
50
25
12.5
6.25
3.125
Peptide concentration (ug/ml)

Fig 1. The CD18 signal peptide analog P1 (aa 1-20) and P5 (aa 5-24) inhibit Lkt-induced cytolysis of BL3 cells. Inhibition of Lkt-induced cytolysis of BL-3 cells by a nested set of peptides (20-mers) spanning the aa 1-291 was tested by the MTT dye-reduction cytotoxicity assay. All data are expressed as mean $\pm$ s.d. (n=3). **(*A*) Inhibition of Lkt-induced cytolysis of BL-3 cells by CD18 peptide analogs.** The peptides were designated by the sequence number of their first aa. MSP-1 represents a 20-mer peptide derived from the major surface protein 1 of *Anaplasma marginale* which was used as the negative control. The peptides were used at a concentration of 100 μg/ml. % Inhibition was calculated as described in the 'materials and methods'. **(*B*) Peptide P5 is a more potent inhibitor of Lkt-induced cytolysis BL-3 cells than peptide P1.** Concentration of peptides P1 and P5 that gives 50% inhibition of Lkt-induced cytolysis of BL-3 cells was determined**.** The peptides P1 and P5 were used in the inhibition assay at concentrations ranging from 3.1 ug/ml to 100 ug/ml. The results subjected to linear regression analysis, are presented with the natural log of the peptide concentration in the X axis and the % inhibition of lysis in the Y axis. **(*C*) Inhibition of Lkt-induced cytolysis of BL-3 cells by the peptide P5 is specific.** Peptides, PSC1 and PSC 2, represent two peptides containing the same aa as the peptide P5, but in a randomly scrambled sequence.

Figure 2

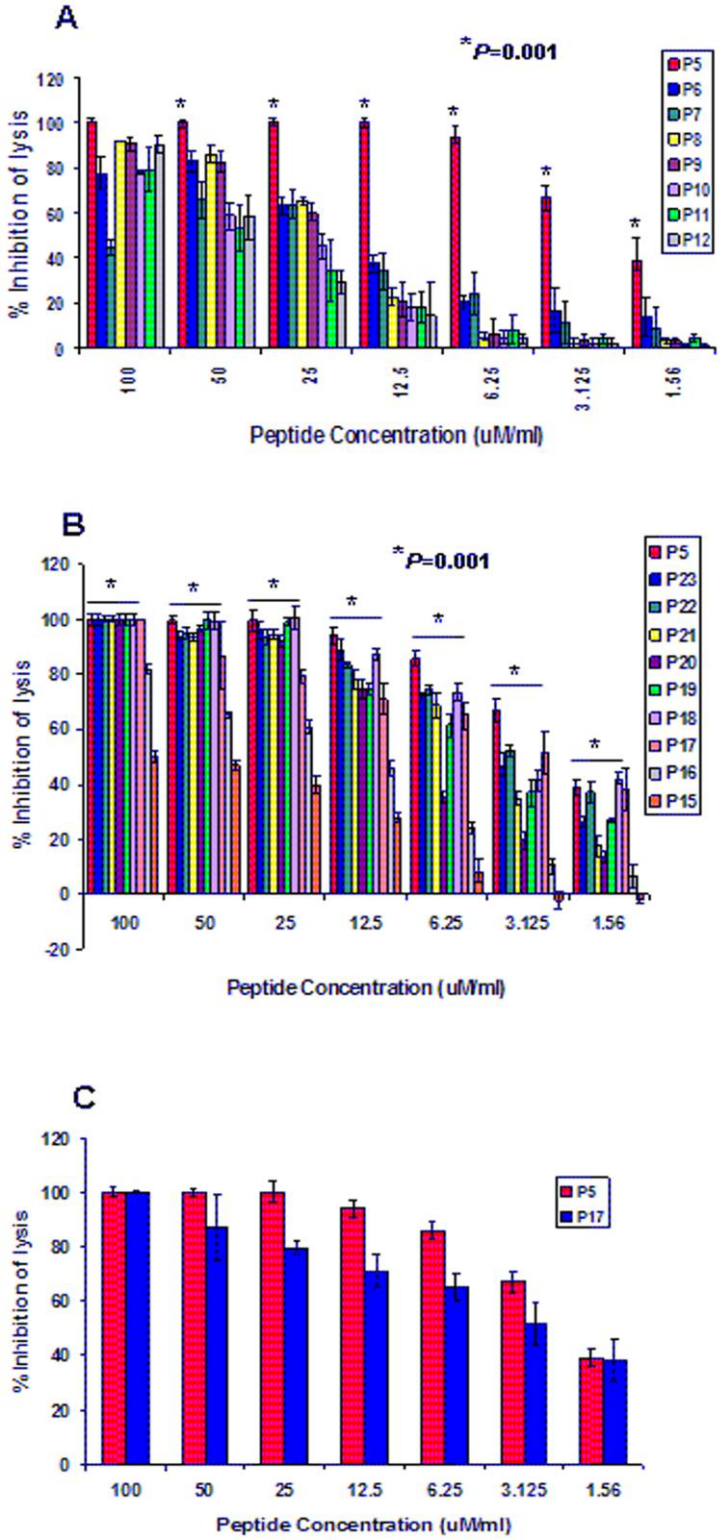
A
*P=0.001
P5
P6
P7
P8
P9
P10
P11
P12
% Inhibition of lysis
Peptide Concentration (uM/ml)
B
*P=0.001
P5
P23
P22
P21
P20
P19
P18
P17
P16
P15
% Inhibition of lysis
Peptide Concentration (uM/ml)
C
P5
P17
% Inhibition of lysis
Peptide Concentration (uM/ml)

Fig. 2. N- and C-terminal truncations of peptide P5 identify aa 5-17 as the Lkt-binding domain on bovine CD18. Inhibition of Lkt-induced cytolysis of BL-3 cells by N-terminally truncated or C-terminally truncated versions of peptide P5 was determined by the MTT dye-reduction cytotoxicity assay. Peptides were used at concentrations ranging from 1.56 to 100 uM/ml. All data are expressed as mean $\pm$ s.d. (n=3). **(*A*) Inhibition of Lkt-induced cytolysis of** BL-3 cells **by peptides with N-terminal truncation.** The peptides are referred to by the sequence number of their first aa: P5 (aa 5-24); P6 (aa 6-24); P7 (aa 7-24); P8 (aa 8-24); P9 (aa 9-24); P10 (aa 10-24); P11 (aa 11-24); P12 (aa 12-24). **(B) Inhibition of Lkt-induced cytolysis of BL-3 cells by peptides with C-terminal truncation.** Results of the inhibition assay in (***A***) above, identified the N-terminal aa of the minimal peptide sequence as aa #5. Therefore, the C-terminally truncated versions of peptide P5 with aa #5 as the N-terminal aa were used in the inhibition of Lkt-induced cytotoxicity assay. These C-terminally truncated peptides are referred to by the position number of their C-terminal aa: P23 (aa 5-23); P22 (aa 5-22); P21 (aa 5-21); P20 (aa 5-20); P19 (aa 5-19); P18 (aa 5-18); P17 (aa 5-17); P16 (aa 5-16); P15 (aa 5-15). But the peptide P5 consists of aa 5-24, as stated under panel A above. **(C) Inhibition of Lkt-induced cytolysis of BL-3 cells by peptides P5 and P17. This figure shows the inhibition of Lkt-induced cytolysis of BL-3 cells by peptides P5 and P17 only. Other peptides are omitted for clarity.** Results of the inhibition assay in (***A***) (***B***) and (*C*) above, identified the minimal peptide sequence of bovine CD18 bound by Lkt as aa 5-17.

Figure 3

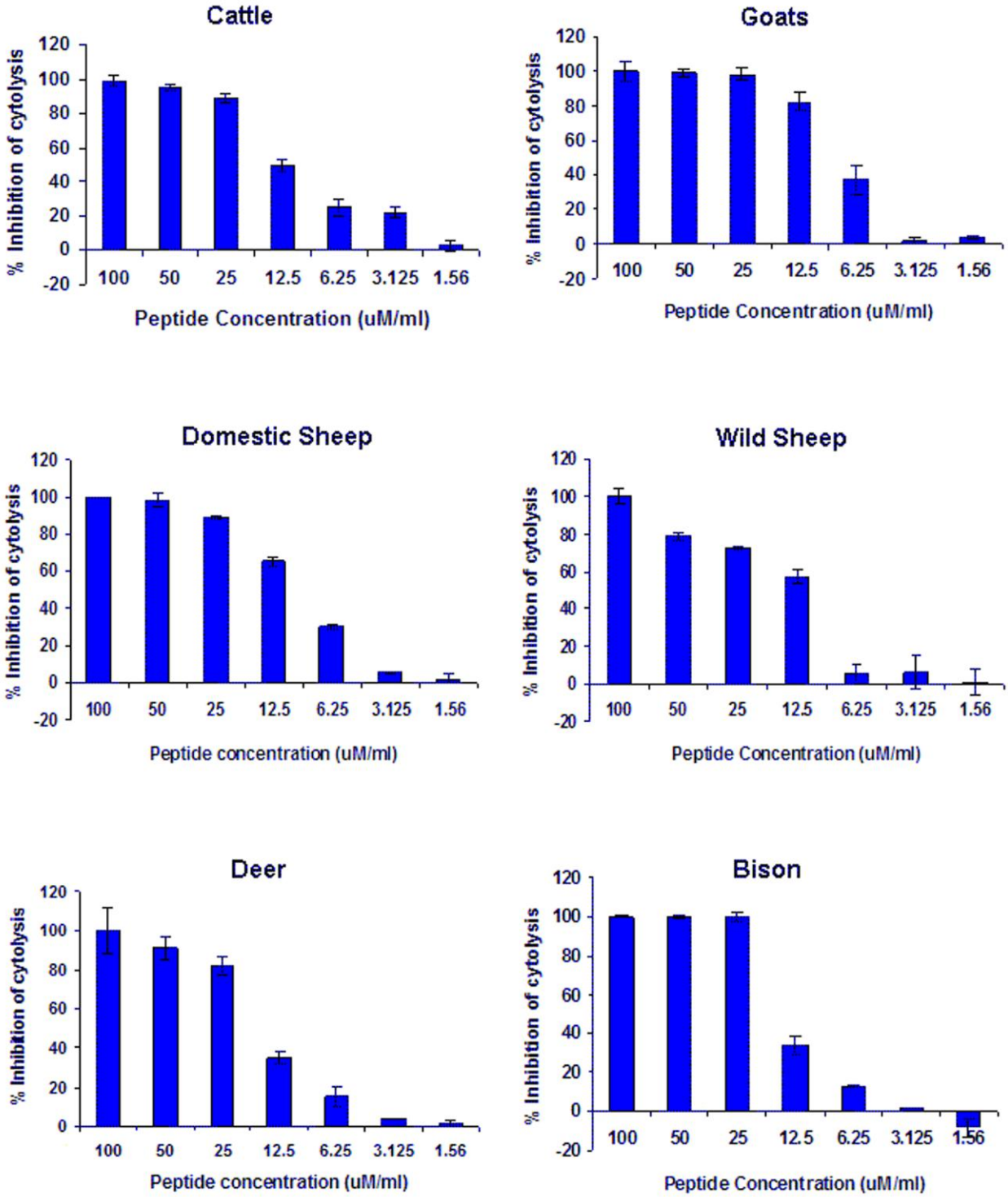
Cattle
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
100 50 25 12.5 6.25 3.125 1.56
Peptide Concentration (uM/ml)
Goats
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
100 50 25 12.5 6.25 3.125 1.56
Peptide Concentration (uM/ml)
Domestic Sheep
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
100 50 25 12.5 6.25 3.125 1.56
Peptide concentration (uM/ml)
Wild Sheep
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
100 50 25 12.5 6.25 3.125 1.56
Peptide Concentration (uM/ml)
Deer
% Inhibition of cytolysis
120
100
80
60
40
20
0
100 50 25 12.5 6.25 3.125 1.56
Peptide concentration (uM/ml)
Bison
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
100 50 25 12.5 6.25 3.125 1.56
Peptide Concentration (uM/ml)

Fig. 3. Inhibition of Lkt-induced cytolysis of ruminant PMNs by peptide P17 (aa 5-17) confirms aa 5-17 as the Lkt-binding domain on CD18 of ruminants. Inhibition of Lkt-induced cytolysis of PMNs of cattle, goats, domestic sheep, wild sheep, deer, and bison was determined by the MTT dye-reduction cytotoxicity assay. Peptides were used at concentrations ranging from 1.56 to 100 uM/ml. All data are expressed as mean $\pm$ s.d. (n=3).

Figure 4

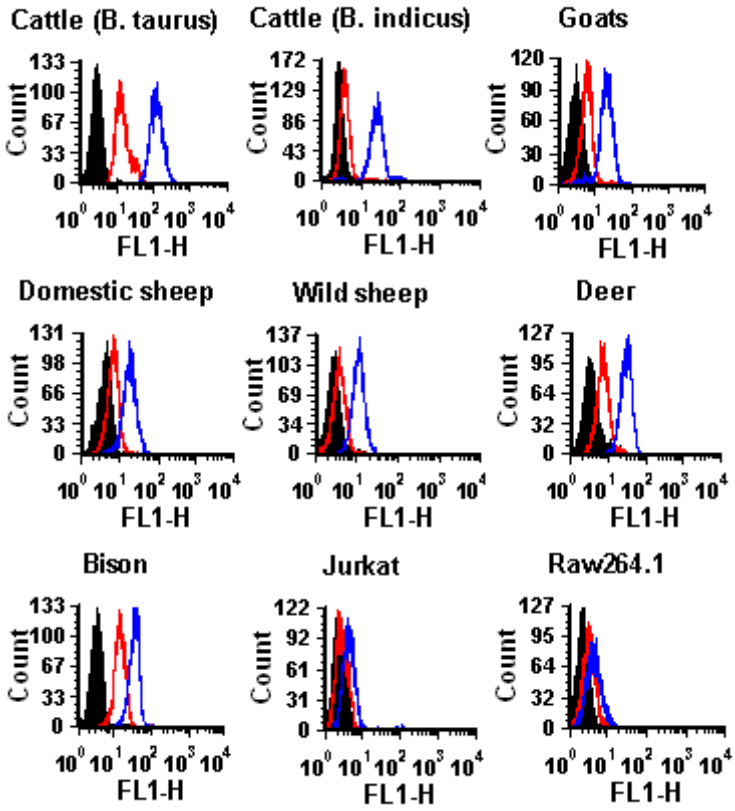
Cattle (B. taurus)
Cattle (B. indicus)
Goats
Domestic sheep
Wild sheep
Deer
Bison
Jurkat
Raw264.1
Count
FL1-H

Fig. 4. Anti-signal peptide serum binds to membrane CD18 of PMNs of all ruminants tested. PMNs were tested by flow cytometry for binding of a chicken anti-serum (1/1,000 dilution) developed against a synthetic peptide spanning aa 5-19 of the signal peptide of bovine CD18. The different panels show the binding of the anti-signal peptide serum to the PMNs of Bos taurus cattle, Bos indicus cattle, goats, domestic sheep, wild sheep, deer, bison, and the cell-lines Jurkat (human T cell leukemia cell line), and RAW264.A (mouse macrophage cell line). Black histogram**:** cells treated with the secondary Ab only; red histogram**:** cells treated with pre-immune chicken serum; blue histogram**:** cells treated with anti-serum against the signal peptide of bovine CD18. The data (10,000 events) were acquired for forward and side scatter using the following settings: FSC, voltage E00 and SSC, voltage 260. Results of one representative experiment out of three are shown.

Figure 5

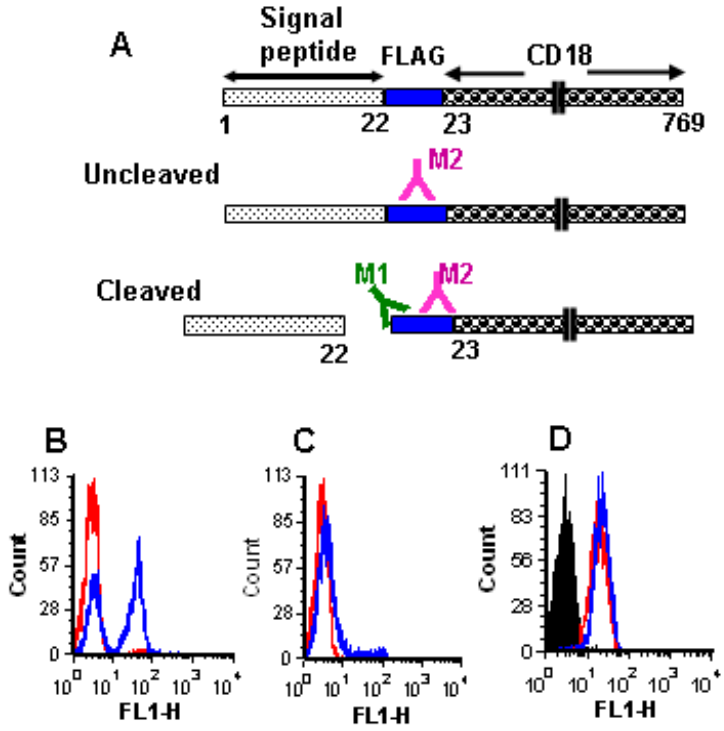
A
Signal peptide
FLAG
CD18
1
22
23
769
Uncleaved
M2
Cleaved
M1
M2
22
23
B
C
D
Count
FL1-H

Fig. 5. The signal peptide of bovine CD18 is not cleaved. (*A*) Introduction of the 'FLAG' epitope at the signal peptide cleavage site and its detection by two MAbs. (*A*) The 'FLAG' epitope introduced at the putative signal peptide cleavage site can be detected by two anti-'FLAG' epitope MAbs M1 and M2. M2 recognizes the FLAG epitope irrespective of its sequence context whereas M1 recognizes only the free N-terminal end of the FLAG epitope (if cleavage occurs). **(*B*) & (*C*) Flow cytometric analysis of expression of the 'FLAG' epitope.** The transfectants 2B2 and BFL were tested for the expression of the 'FLAG' epitope with the MAb M2 **(*B*)** and M1 **(*C*)**. In both panels B and C, the red and the blue histograms represent 2B2 and BFL, respectively. **(*D*) Flow cytometric analysis of bovine CD18 expression.** The untransfected parent cells P815 (black histogram), or P815 cells transfected with either bovine CD18 (2B2; red histogram) or bovine CD18 containing the 'FLAG' epitope at the cleavage site (BFL; blue histogram), were tested by flow cytometry for expression of bovine CD18 with an anti-bovine CD18 MAb. Results of one representative experiment out of three are shown.

Figure 6

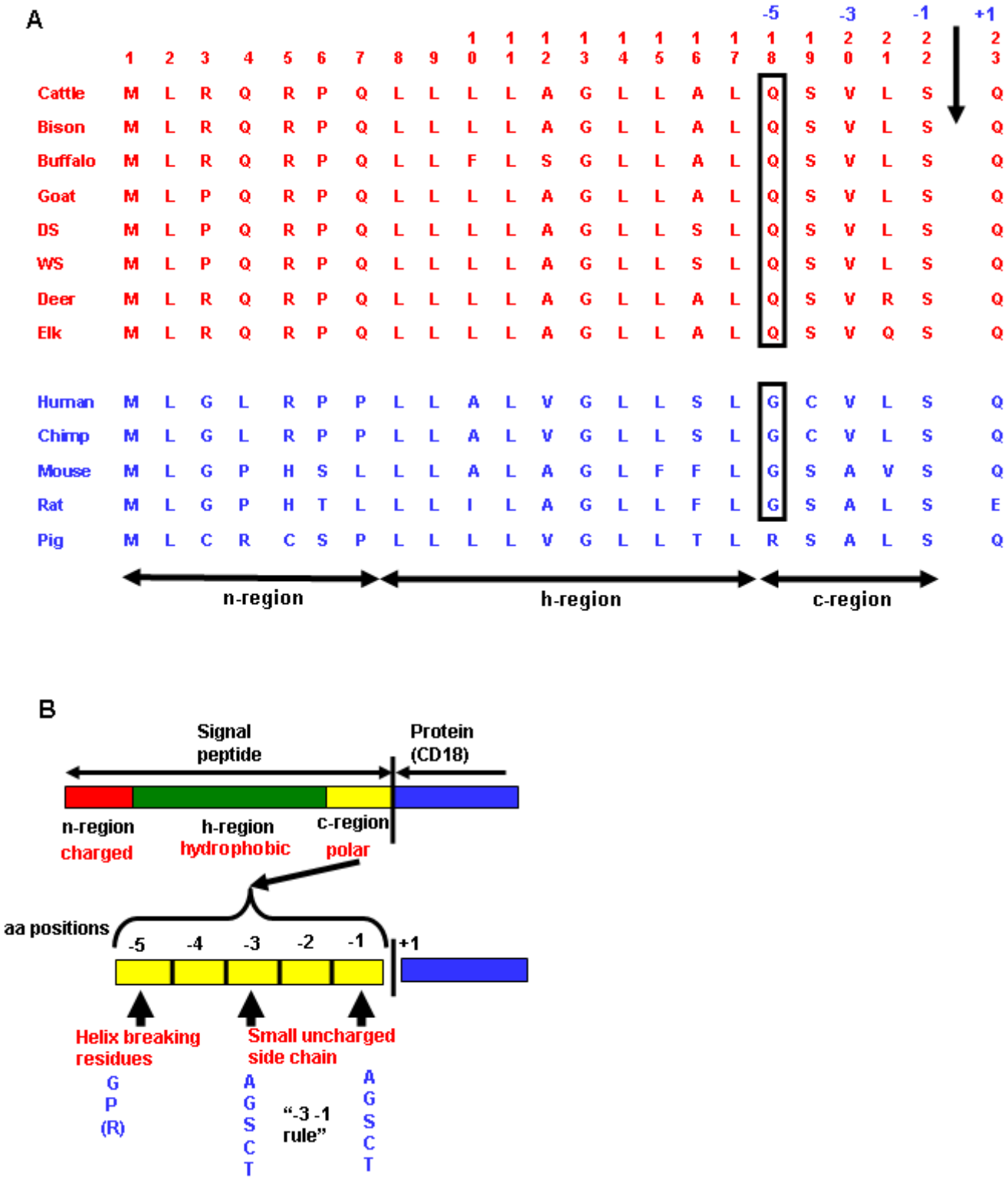
A
-5
-3
-1
+1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Cattle M L R Q R P Q L L L L A G L L A L Q S V L S Q
Bison M L R Q R P Q L L L L A G L L A L Q S V L S Q
Buffalo M L R Q R P Q L L F L S G L L A L Q S V L S Q
Goat M L P Q R P Q L L L L A G L L A L Q S V L S Q
DS M L P Q R P Q L L L L A G L L S L Q S V L S Q
WS M L P Q R P Q L L L L A G L L S L Q S V L S Q
Deer M L R Q R P Q L L L L A G L L A L Q S V R S Q
Elk M L R Q R P Q L L L L A G L L A L Q S V Q S Q
Human M L G L R P P L L A L V G L L S L G C V L S Q
Chimp M L G L R P P L L A L V G L L S L G C V L S Q
Mouse M L G P H S L L L A L A G L F F L G S A V S Q
Rat M L G P H T L L L I L A G L L F L G S A L S E
Pig M L C R C S P L L L L V G L L T L R S A L S Q
n-region
h-region
c-region
B
Signal peptide
Protein (CD18)
n-region
charged
h-region
hydrophobic
c-region
polar
aa positions
-5 -4 -3 -2 -1 +1
Helix breaking residues
G
P
(R)
Small uncharged side chain
A G S C T
"-3 -1 rule"
A G S C T

Fig. 6. The signal peptide of CD18 of ruminants contains cleavage-inhibiting glutamine (Q) at aa position -5 relative to the cleavage site, whereas that of non-ruminants contain cleavage-conducive glycine (G). (*A*) Comparison of signal peptide sequences of ruminants and non-ruminants (GenBank Accession #: cattle: M81233; bison: EU553919; buffalo: AY842449; goat: AY452481; domestic sheep: DQ470837; wild sheep: DQ104444; deer: EU623794; elk: EU553918; human: NM0002211; chimpanzee: NM001034122; mouse: X14951; rat: NM001037780; pig: U13941). DS, WS and Chimp represent domestic sheep, wild sheep and chimpanzee, respectively. The arrow indicates the signal peptide cleavage site. **(*B*) The "-3,-1, rule" for cleavage of signal peptides [43].**

Figure 7

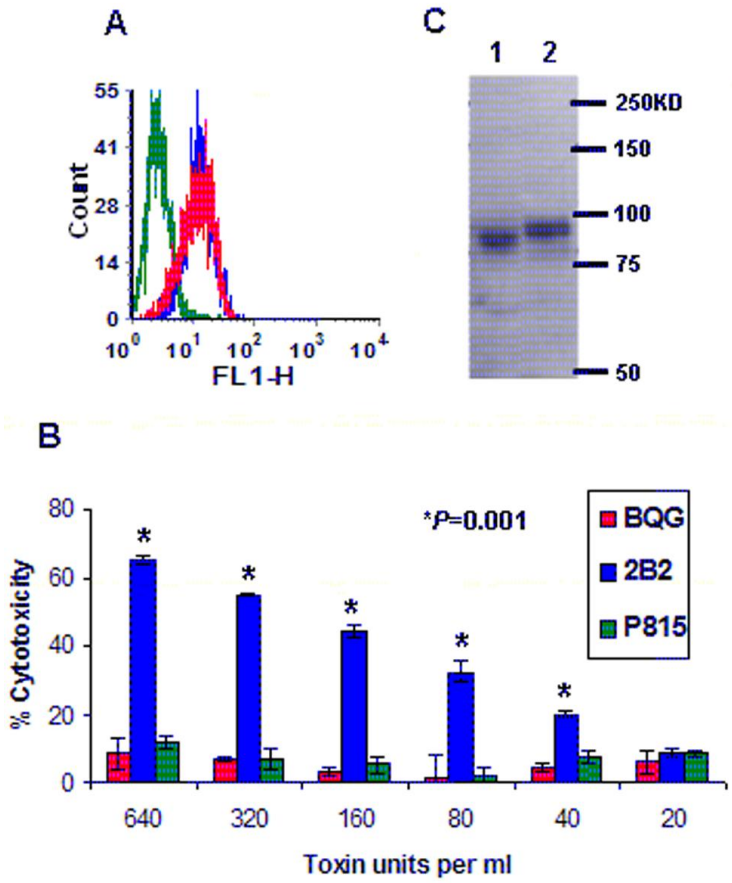
A
55
41
28
14
0
Count
10^0 10^1 10^2 10^3 10^4
FL1-H
C
1 2
250KD
150
100
75
50
B
80
60
40
20
0
% Cytotoxicity
*P=0.001
BQG
2B2
P815
640 320 160 80 40 20
Toxin units per ml

Fig. 7. Mutation of glutamine (Q) to glycine (G) at position -5 of the signal peptide of bovine CD18 abrogates Lkt-induced cytolysis of transfectants expressing CD18 with Q(-5)G mutation. (*A*) Transfectants 2B2 and BQG express similar levels of CD18. Transfectants expressing bovine CD18 (2B2, blue histogram), or bovine CD18 containing the Q(-5)G mutation (BQG, red histogram), and the parent cells (P815, green histogram) were tested for expression of bovine CD18 by flow cytometric analysis with an anti-bovine CD18 MAb. Results of one representative experiment out of three are shown. **(*B*) Transfectant 2B2 is lysed by Lkt in a concentration dependent manner whereas BQG is not.** Transfectants 2B2, BQG, and P815 cells were tested for susceptibility to Lkt-induced cytolysis by the MTT dye-reduction cytotoxicity assay. All data are expressed as mean $\pm$ s.d. (n=3). **(*C*) CD18 of transfectant BQG exhibits a lower molecular weight than that of 2B2.** Cell lysate from the transfectant 2B2 (expressing wild type CD18) and BQG (expressing CD18 with Q(-5)G mutation) were subjected to SDS-PAGE (8% gel) followed by western blot analysis with the anti-CD18 MAb, BAQ30A. Lane 1: Lysate from the transfectant BQG. Lane 2: lysate from the transfectant 2B2.

Figure 8

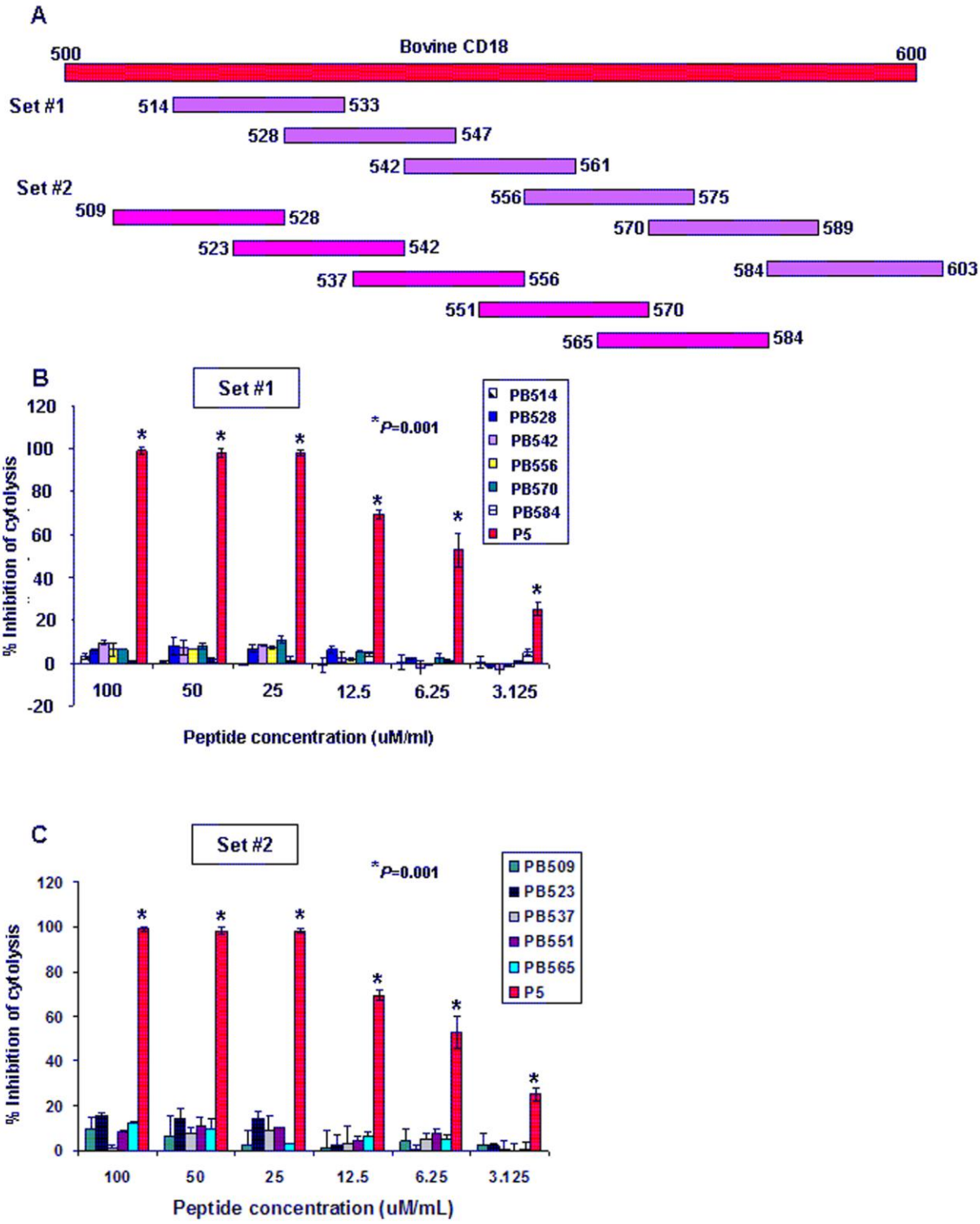
A
Bovine CD18
500
600
Set #1
514
533
528
547
542
561
Set #2
556
575
509
528
570
589
523
542
584
603
537
556
551
570
565
584
B
Set #1
PB514
PB528
PB542
PB556
PB570
PB584
P5
*P=0.001
% Inhibition of cytolysis
120
100
80
60
40
20
0
-20
100
50
25
12.5
6.25
3.125
Peptide concentration (uM/ml)
C
Set #2
PB509
PB523
PB537
PB551
PB565
P5
*P=0.001
% Inhibition of cytolysis
120
100
80
60
40
20
0
100
50
25
12.5
6.25
3.125
Peptide concentration (uM/mL)

Fig. 8. Peptides spanning aa 500-600 of bovine CD18 fail to inhibit Lkt-induced cytolysis of bovine PMNs. (*A*). Nested set of peptides spanning aa 500-600. One set (#1) of peptides (20mer) spanning aa 500-600 were synthesized and tested in the inhibition assay. Since none of the peptides inhibited Lkt-induced cytolysis of bovine PMNs, another set (#2) of peptides with points of origin differing from that of the first set were synthesized. **(*B*) & (*C*).** Inhibition of Lkt-induced cytolysis of bovine PMNs by the two sets of peptides was tested by the MTT dye-reduction cytotoxicity assay. All data are expressed as mean $\pm$ s.d. (n=3).

CHAPTER THREE

Effect of endobronchial inoculation of a peptide analog of CD18 on *M. haemolytica*-caused pneumonia in a calf challenge model

Sudarvili Shanthalingam[1], Rohana P. Dassanayake[1], Renuka Subramaniam[1], Kathleen A Potter[1], Gary J Haldorson[1], Steven M. Parish[2], Dan Righter[1], Kathleen, Donald P. Knowles[3], and Subramaniam Srikumaran[1*]

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ABSTRACT

Leukotoxin (Lkt) produced by *Mannheimia haemolytica* is the major virulence factor of this organism. Lkt-induced cytolysis and degranulation of alveolar macrophages and polymorphonuclear leukocytes is responsible for the acute inflammation and lung injury characteristic of pneumonia caused by *M. haemolytica*. Previously, we identified a peptide analog of CD18 (P17, spanning amino acids 5-17) that effectively inhibits Lkt-induced cytolysis of ruminant leukocytes in *in vitro* cytotoxicity assays (Shanthalingam and Srikumaran, 2009). The objective of this 'proof of concept' study was to determine the ability of this peptide to inhibit or mitigate lung lesions in a calf challenge model of *M. haemolytica*. Three groups of four calves each were inoculated endobronchially with logarithmic phase cultures of *M. haemolytica* ($5x10^9$ CFU per 10 ml of culture medium) alone (Group I), or along with a control peptide (Group II), or with the CD18 peptide analog P17 (Group III). Animals were observed for clinical signs at different time points, euthanized at 90 hours post-inoculation, and necropsied. The total clinical disease scores for Group III calves were not different for group I and II. All the calves presented gross pulmonary lesions consistent with fibrinonecrotic pneumonia characteristic of *M. haemolytica* infection. The difference in percent volume of lungs exhibiting gross pneumonic lesions among the three groups was not statistically significant ($P = 0.9$). However, *M. haemolytica* isolated from the lungs of Group III calves was 100- to 1000-fold less than those isolated from the calves in Group I and Group II. This difference, expressed as CFU of *M. haemolytica* per gram of lung tissue ($P < 0.001$) suggesting that the CD18 peptide analog reduced leukotoxic activity in the lungs enabling more effective bacterial clearance by the phagocytes. We propose that prolonging the presence and activity of the CD18 peptide analog in the lungs by

using a nanoparticle delivery system such as crystallized dextran microspheres should enhance its protective ability.

INTRODUCTION

Mannheimia (Pasteurella) *haemolytica* is the primary bacterial pathogen of bovine pneumonic pasteurellosis, an economically important disease of beef and dairy cattle in North America and Western Europe (Frank, 1989; Whiteley et al., 1992). The greatest impact of pasteurellosis occurs in recently weaned beef calves shortly after entry into feedlots (Jubb and Kennedy, 1970; Mosier et al., 1989; Wilson, 1989). This organism resides in the nasopharynx and tonsils of healthy calves as a commensal bacterium. In conjunction with active viral infection and stress factors it multiplies rapidly, reaches the lung and causes an acute fibrinonecrotic pleuropneumonia. *M. haemolytica* serotype 2 is commonly found in the upper respiratory tract of healthy cattle, but following viral infection or stress, is rapidly replaced by serotype 1 (Frank and Smith 1983, Frank, 1979, 1988; Gonzalez and Maheswaran, 1993).

M. haemolytica produces several virulence factors which include the capsule, outer membrane proteins, neuraminidase, adhesins, lipopolysaccharide (LPS), and an exotoxin known as leukotoxin (Lkt; Confer et al., 1990). Based on the fact that Lkt-deletion mutants cause reduced mortality and much milder lung lesions than the wild-type organisms, Lkt has been widely accepted as the most important virulence factor (Petras et al., 1995; Tatum et al., 1998; Highlander et al., 2000; Dassanayake et al., 2009). It is a member of the RTX (repeats in toxin) family of toxins produced by a number of Gram negative bacteria, which includes the *Escherichia coli* hemolysin (HlyA), *Bordetella pertussis* adenylate cyclase/hemolysin (CyaA), and the *Actinobacillus pleuropneumoniae* Apx toxins (Welch et al., 1995). Lkt produced by *M. haemolytica* is a Ca-dependent pore-forming cytolysin (Coote, 1992) which is specific for ruminant leukocytes and platelets (Kaehler et al., 1980; Shewen and Wilkie, 1982; Clinkenbeard and Upton; 1991). At high concentrations, Lkt, like other RTX members, creates pores in the cell

membranes of leukocytes that lead to K^+ efflux, Ca^{2+} influx, cell swelling and eventual lysis. Cytolysis of both alveolar macrophages and polymorpho nuclear leukocytes (PMNs) reduces the bacterial clearance from the respiratory tract (Maheswaran et al., 1992). Moreover, phagocytic cell lysis causes the release of several lysosomal proteolytic enzymes, reactive oxygen intermediates and fibrinogen which cause structural degradation of the alveolar lining (Hochberg, 1989; Watson et al., 1995). Hence, the interaction of Lkt with alveolar macrophages and neutrophils is responsible for the acute inflammation and subsequent lung injury characteristic of this disease. Therefore abrogation of Lkt-leukocyte interaction could prevent or mitigate the lung lesions in *M. haemolytica*-caused pneumonia.

Earlier studies by us and others (Wang et al., 1998; Ambagala et al., 1999, Li et al., 1999; Jayaseelan et al., 2000) identified β_2 integrins as the receptor for Lkt of *M. haemolytica* on bovine leukocytes. β_2-integrins are leukocyte specific integrins, which are heterodimeric molecules composed of two non-covalently associated subunits, α (CD11) and β (CD18). CD18 associates with four distinct α chains to give rise to four different β_2-integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (CR4), and CD11d/CD18 (Gahmberg et al, 1998; and Noti et al, 2000). Studies in our laboratory have demonstrated that CD18, the β subunit of β_2 integrins, is the functional receptor for Lkt (Deshpande et al., 2002; Liu et al., 2007; Dassanayake et al., 2007a, 2007b). Furthermore, we have mapped the Lkt binding site on bovine CD18 to lie between amino acids 1-291 (Gopinath et al., 2005). In a recent study, using synthetic peptides spanning amino acids 1-291 of bovine CD18 in inhibition of Lkt-induced cytolysis assays, we determined that the precise binding site of Lkt is formed by amino acids 5-17 of CD18 (Shanthalingam and Srikumaran, 2009). Binding of a ligand to its receptor can be potentially blocked by synthetic peptides representing the amino acids involved in the binding (Tibbetts et al., 1999, 2000).

Therefore the objective of this study was to determine the ability of the peptide spanning amino acids 5-17 of bovine CD18 to inhibit or mitigate the disease caused by *M. haemolytica* in a calf challenge model. Here we report the results of a preliminary 'proof of concept' calf infection study in which the CD18 peptide analog containing amino acids 5-17 was co-administered with *M. haemolytica*.

MATERIALS AND METHODS

Preparation of *M. haemolytica* inoculum for endobronchial challenge: *M. haemolytica* serotype-1 strain SH789, isolated from the pneumonic lung of a calf, was streaked on blood agar plate and incubated overnight at 37 ^{0}C. The following day few colonies were transferred to 3 ml of pre-warmed brain heart infusion (BHI) broth and incubated for 3 hours at 37 ^{0}C with constant shaking (200 cycles/minute). Two BHI agar plates were 'lawned' with this bacterial culture using sterile cotton swabs and incubated overnight at 37 ^{0}C. The following day (day of inoculation) *M. haemolytica* was harvested from the BHI agar plates and transferred to 40 ml of pre-warmed BHI broth in a 250 ml flask, and incubated for 2.5 hours at 37 ^{0}C with constant shaking to obtain cultures in the logarithmic phase of growth. The culture was centrifuged at 6000 X g at 20 ^{0}C for 30 minutes and the pellet was washed once with RPMI 1640 (without phenol red) medium. The bacterial pellet was re-suspended in 4 ml of RPMI 1640 (without phenol red), and 1 ml of this culture was added to 50 ml of pre-warmed RPMI 1640 (without phenol red) containing L-glutamine (1 ml L-glutamine/100 ml RPMI) in a 250 ml flask. Bacteria were incubated for 3 hours at 37 ^{0}C with constant shaking to obtain logarithmic phase culture and the optical density (OD) was measured. The culture was appropriately diluted to obtain a concentration of 1 X 10^9 CFU of

M. haemolytica per ml. Five ml of this preparation per calf was used for endobronchial challenge. The bacterial concentration was confirmed the following day by culturing diluted aliquots of the inoculum on BHI agar and counting the resulting colonies.

Peptides: The peptide (P17) containing amino acids 5-17 of bovine CD18 (NH_2-RPQLLLLAGLLAL-OH), and the peptide (PSC) containing the same amino acids as peptide P17 but in a randomly scrambled sequence (NH_2-LRALLPLQLLAGL-OH), were synthesized at Neopeptide (Cambridge, MA). Both peptides were re-suspended in dimethysulfoxide (ATCC) at a concentration of 20 mg/ml and stored at -20 ^{0}C until used. Based on the results of *in vitro* neutralization of Lkt by peptide P17, calves of group II and III were endobronchially inoculated with 2 mg of peptide in 5 ml of RPMI mixed with 5 X 10^9 CFU of *M. haemolytica* in 5 ml of RPMI.

In vitro neutralization of Lkt: Five ml aliquots of *M. haemolytica* containing 1 X 10^9 CFU /ml of RPMI 1640 were mixed with 5 ml aliquots of the peptide (P17 or PSC) at a concentration of 5, 4, or 2 mg per 5 ml of RPMI 1640 (without phenol red), and incubated for 4 – 5 hours at 37 ^{0}C with constant shaking. The bacteria were removed from the culture by centrifugation (13, 500 X g for 20 min at 4 ^{0}C), and the supernatant fluid was filter-sterilized and stored at -20 ^{0}C until tested by the cytotoxicity assay for leukotoxic activity.

Detection of Lkt-induced cytolysis of target cells: The MTT [3-(4,5-dimethylthiazoyl-2-YI)-2,5-diphenyl tetrazolium bromide; Sigma] dye reduction cytotoxicity assay for detection of Lkt-induced cytolysis of target cells has been previously described by us (Gentry and Srikumaran,

1991). This assay measures the ability of the ER-resident enzymes in viable cells to convert a tetrazolium dye into a purple formazan precipitate, which is later dissolved in acid isopropanol. The optical density (OD) of the end product, representing the intensity of the purple color developed, is directly proportional to the viability of the cells. Briefly, the target cells were re-suspended in RPMI 1640 (without phenol red) at a concentration of 5 X 10^6 cells ml^{-1}, and seeded into 96 well round bottom microtiter plates (50 ul/well) containing the serially diluted Lkt in duplicates and incubated at 37 ^{0}C for 1 hour. Cells were centrifuged at 600 X g for 5 min following incubation, and the supernatant fluid was discarded. The cell pellets were re-suspended in 100 ul of RPMI 1640 without phenol red and 20 ul of 0.5% MTT dye were added to each well. After 1 hour of incubation at 37 ^{0}C, the plates were centrifuged at 600 X g for 5 min and the supernatant fluid was removed. The formazan precipitate was thoroughly dissolved in 100 ul acid isopropanol and the OD of the samples was measured using an ELISA reader at 540 nm. The percent cytotoxicity was calculated as follows: % cytotoxicity = [1-(OD of toxin-treated cells / OD of toxin-untreated cells)] X 100.

Detection of inhibition of Lkt-induced cytolysis of target cells by the Lkt-neutralizing Abs in serum. For Lkt neutralization, 50 ul of toxin preparation at a 50 % toxicity end point titer of 20 Units/ml was incubated with 50 ul of serum (2 fold dilutions starting at 1:20) at 4 ^{0}C for 1 hour (Shanthalingam and Srikumaran, 2009). Bovine lymphoma cells (BL3; 5 X 10^6 /ml) were added, and the MTT assay was performed as described above. The percent inhibition of cytolysis was calculated as follows: % Inhibition of cytolysis = [1 – (% cytolysis in the presence of serum / % cytolysis in the absence of serum)] x 100.

Animal inoculation: All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington State University before the onset of the study. Twelve Holstein calves were randomly assigned to three experimental groups. Calves were matched for age when assigned into three groups. Prior to inoculation, serum samples and pharyngeal and nasal swabs were collected from all the calves. Group I calves received endobronchial injections of 5 X 10^9 CFU of *M. haemolytica* in 10 ml of RPMI. Group II calves received 5 X 10^9 CFU of *M. haemolytica* and 2 mg of peptide PSC in 10 ml of RPMI. Group III calves received 5 X 10^9 CFU of *M. haemolytica* and 2 mg of peptide P17 in 10 ml of RPMI. The inoculum was flushed down with an additional 10 ml of RPMI in all calves. Clinical disease in each calf was scored at different time points post-inoculation. Calves were humanely euthanized 90 hours post-inoculation, and the percent volume of lungs exhibiting gross pulmonary pathology was calculated using morphometric methods.

Scoring of clinical disease: Physical examination of each calf was performed immediately prior to experimental infection and at 6, 18, 24, 42, 48, 66, 72 and 90 hours post-inoculation. Signs of clinical disease were allocated points according to the scoring system followed by Malazdrewich et al (2003; Table 1).

Serotyping: *M. haemolytica* isolated from pharyngeal and nasal swabs prior to inoculation and from lung tissue at necropsy, were typed using anti-serotype A1 serum (kindly provided by Dr. Robert Briggs, National Animal Disease Center, Ames, IA). One milliliter of fresh culture was centrifuged at 6800 X g for 3 minutes and the pellet was re-suspended in 100 ul of Hanks' balanced salt solution (HBSS) medium containing 0.25% of bovine serum albumin (BSA).

Twenty-five ul of culture was then placed on the agglutination plate. Anti-A1 specific serum was diluted in HBSS medium containing 0.25% of BSA and 25 ul of the diluted serum at 1/16 dilution was added to the bacteria and mixed by gentle rocking of the plate. The reference A1 strain and culture media were used as the positive and negative controls, respectively.

Quantitation of gross pulmonary pathology: On necropsy lungs were removed and gross pneumonic lesion development in each lobe of lung was observed. The entire lung from each animal was sliced at 1 cm thickness and the total and pneumonic lesion areas were traced onto transparent acetate sheets. The traced portions were scanned into ImgaeJ, NIH Image (National Institute of Health; Nethesda, MD), which was used to measure areas representing both the total serial section and the gross pneumonic lesions within it. Measured areas for each serial section were used to calculate the volume of each lung and the grossly pneumonic regions within it using Simpson's rule: $V = (1/3)\ h\ [(A_0 + A_n) + 4(A_1 + A_3 + \ldots + A_{n-1}) + 2\ (A_2 + A_4 + \ldots\ldots + A_{n-2})]$ where V is the total or pneumonic lung volume, h is the thickness of each slice in centimeters, and A, A_0, A_1, A_2, A_n represent measured lung or pneumonic lesion areas for lung slices 0, 1, 2, n. These values were then used to calculate the percent volume of the lung exhibiting gross pulmonary pathology in each calf.

Lung tissues from representative gross lesions in each calf were collected for histopathological evaluation. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin using standard techniques. After routine processing, 5 um tissue sections were stained with hematoxylin and eosin and used for subjective, non-quantitative histopathological examination.

Re-isolation of *M. haemolytica* from pneumonic lungs: Using aseptic techniques, fresh lung samples and pharyngeal and nasal swabs were obtained for isolation and characterization of bacteria including *M. haemolytica* and other *Pasteurella* species. Serotyping of *M. haemolytica* isolates were performed using anti-serotype A1 specific sera.

Bacteriological examination: Small samples of tissue (1g) were obtained from affected regions from the same lobe. The samples were homogenized into 3 mls of RPMI 1640 and diluted 10-fold ($1 \text{ X } 10^{-1}$ to $1 \text{ X } 10^{-6}$). Ten ul aliquots of each dilution were applied to BHI agar plates on 5 spots and incubated at $37\ ^{0}$C overnight and viable counts were determined. Representative colonies were checked for *M. haemolytica* by colony PCR. Primers used were as follows: forward 5`-AGAGGCCAATCTGCAAACCTC-3` and reverse 5`-GTTCGTATTGCCCAACGCCG-3`. Counts were expressed as CFU of *M. haemolytica* /g of lung tissue.

Statistical analysis

Clinical scores and the percent volume of the lung exhibiting gross pneumonic lesions were expressed as the mean $\pm$ SEM. Clinical scores between the groups were compared using repeated measure analysis of variance tests (ANOVA). Pneumonic lung scores and the quantity of *M. haemolytica* / g of lung tissue of all three groups were also compared using one-way ANOVA. Differences were considered significant at a value of $P < 0.05$.

RESULTS

Co-incubation of peptide P17 with *in vitro* cultures of *M. haemolytica* abrogates leukotoxic activity.

Before proceeding with the *in vivo* experiments, the inhibitory effect of the peptide P17 on the leukotoxic activity of *in vitro* cultures of *M. haemolytica* was determined. *M. haemolytica* cultures were incubated with 2, 4, or 5 mg of peptide P17 or the control peptide PSC, and the leukotoxic activity in the culture supernatant fluids was determined by the dye-reduction cytotoxicity assay. The supernatant fluids from *M. haemolytica* cultures incubated with the peptide P17 did not exhibit significant leukotoxic activity whereas those from cultures incubated with the control peptides had significant leukotoxic activity (Fig 1). The difference in % cytotoxicity exhibited by the supernatant fluids from the cultures incubated with the peptide P17 and PSC was statistically significant ($P < 0.001$). Inhibition of cytotoxicity did not decrease when the quantity of peptide P17 was reduced from 5 to 4 and 2 mg, indicating that the peptide P17 can inhibit the Lkt-induced cytolysis of target cells even at 2 mg (per 5 X 10^9 CFU of *M. haemolytica*).

Pre-inoculation status of calves (nasopharyngeal flora and anti-Lkt antibodies)

The results of bacterial isolation are summarized in Table 2. Ten out of 12 calves carried *M. haemolytica* in their pharynx whereas only 4 calves carried it in the nasal cavity. None of the isolates belonged to serotype 1, the serotype of *M. haemolytica* used for inoculation. The unavailability of antisera specific for all known serotypes of *M. haemolytica* prevented us from identifying the precise serotype of these bacteria. *Pasteurella multocida* and *Bibersternia trehalosi* were isolated from two and three calves, respectively. All the calves used in the experiment had

low titers of Lkt-neutralizing antibodies as revealed by the cytotoxicity inhibition assay. The titers ranged from 20 to 320.

Clinical disease scores

Physical examination of each calf was conducted immediately prior to experimental infection and 6, 18, 24, 42, 48, 66, 72 and 90 hours post-infection. All calves were clinically normal (clinical score = 0) pre-inoculation. Within 6 hours of infection, all calves developed clinical signs of disease. Rectal temperature increased to 105-106 ^{0}F within 6 hours post-inoculation and returned to baseline 24 hours post-inoculation as previously reported by other workers (Corrigan et al., 2007). All calves in Groups I and II had nasal discharge throughout the study period, but the Group III calves had nasal discharge only up to 24 hours post-inoculation. The total observational disease scores for Group III calves (peptide P17) were similar to that of other two groups I and II.

Gross lesions

All the calves presented gross pulmonary lesions consistent with fibrinonecrotic pneumonia characteristic of *M haemolytica*-caused pneumonia. The pulmonary lesions in all calves were qualitatively similar but differed in severity and extent. Affected lung tissue exhibited consolidation, congestion, and prominent interlobular septae due to fibrin deposition. These lesions were mainly present in the right lung and to a limited extent in the left lung (Fig 3A). The percent volume of the lungs exhibiting gross pneumonic lesions, as determined by morphometic techniques (Malazdrewich, et al., 2004) are shown in Table 4. The difference in the percent

volume of lungs exhibiting gross pneumonic lesions among the three groups was not statistically significant ($P = 0.9$).

Histopathological examination of pulmonary tissues from the calves revealed that the lesions observed in the calves were characteristic of pneumonia caused by *M. haemolytica*. Interlobular septa were markedly widened by fibrin and fribrous tissue. Within lobules, discreet foci of parenchymal necrosis were outlined by dense bands of degenerate neutrophils, often with streaming nuclei ('oat cells'). Within necrotic foci, alveolar walls were lysed and alveolar spaces were filled with fibrin, red blood cells and nuclear debris. In some calves large colonies of coccobacilli were present within affected areas. Parenchyma adjacent to necrotic foci was either collapsed or filled with fibrin and macrophages (Fig 3B).

Re-isolation of *M. haemolytica* serotype 1 from pneumonic lungs of calves

Pure cultures of bacteria (*M. haemolytica*) were recovered from the pneumonic lungs of all calves. All isolates were identified as *M. haemolytica* by PCR, and confirmed as serotype-1 by serotyping analysis. All heart blood cultures were negative for *M. haemolytica*, indicating that the infection was confined to the respiratory tract. *M. haemolytica* were isolated from pharynx and nasal cavity of most of the calves (Table 2). All the isolates from pharynx belonged to serotype 1 but the isolates from nasal cavity were not. Isolation of *M. haemolytica* from the lungs revealed that the calves in Group III carried approximately 100- to 1000-fold less organisms in the lungs than the calves in Group I and Group II (Table 4). This difference, expressed as CFU of *M. haemolytica* per g of lung tissue, was statistically significant ($P < 0.001$).

DISCUSSION

M. haemolytica Lkt-induced cytolysis and degranulation of macrophages and PMNs is responsible for the acute inflammation and lung injury that is characteristic of pneumonia caused by this organism. It is conceivable that abrogation of Lkt-induced cytolysis could prevent or mitigate the lung lesion. Previously, we have shown that a peptide representing the amino acid sequence of Lkt-binding site on its receptor, CD18, effectively inhibits the Lkt-induced cytolysis of target cells (Shanthalingam and Srikumaran, 2009). In this study we have confirmed that this peptide analog of CD18 abrogates the leukotoxic activity of *in vitro* cultures of *M. haemolytica* (Fig 1) which prompted us to test the efficacy of this peptide in a calf challenge model.

M. haemolytica serotype 1 was the obvious choice for this study since it is the serotype that predominantly causes pneumonia in cattle although other serotypes such as 2, 4 and 7 commonly inhabits the nasopharynx of healthy cattle (Frank and Smith 1983, Frank, 1988; Gonzalez and Maheswaran, 1993). All the pharyngeal isolates obtained from the calves pre-inoculation belonged to serotypes other than serotype 1 allowing us to track the inoculated *M. haemolytica* by serotyping. The presence of Lkt-neutralizing antibodies in the serum suggests that these calves would have had these antibodies in the alveolar epithelial lining fluid which may have had an effect on the bacterial clearance. Although the calves were matched for Lkt-neutralizing antibody titers, we realize that it is not the ideal situation. In future experiments, we will use colostrum-deprived calves to eliminate any effect due to Lkt-neutralizing antibodies.

All the calves developed high rectal temperature within 6 hours of inoculation which dropped to normal levels in 24 hours. LPS represents 10 to 25% of the dry weight of *M. haemolytica* bacteria (Keiss et al., 1964) and it forms high-molecular-weight aggregates with Lkt (Li and Clinckenbeard, 1999). Since LPS stimulates alveolar macrophages to produce TNFα and

interleukin-8, leading to inflammation, it is likely that some of the effects that we observed were LPS related. The total observational disease scores for Group III calves were not significantly lower than those for group I and II. It is likely that the peptide was absorbed from the surface of the respiratory epithelium, which could have resulted in the loss of protective effect of the peptide. Use of peptides absorbed to solid particles which slowly release the peptide is likely to prolong the protective effect of the peptides which could be expected to mitigate the disease.

The peptide P17 strongly inhibited Lkt-induced cytolysis of bovine PMNs in *in vitro* assays. It is surprising that the gross pneumonic lesions in calves inoculated with this peptide and *M. haemolytica* (Group III) were no less than those in calves inoculated with the control peptide PSC and *M. haemolytica* (Group II), or *M. haemolytica* alone (Group I). The failure of the peptide to mitigate lung lesions could be due to (1) inadequate quantity of the peptide; (2) rapid absorption of the peptide from the lung epithelial surface; or (3) degradation of the peptide by proteolytic enzymes (4) artifact of challenge model (fixed volume of inoculum). Again, use of peptides adsorbed on solid particles which slowly release the peptide is likely to prolong the protective effect of the peptides which could be expected to mitigate the lung lesions (Freiberg and Zhu, 2004; Schroder and Stahl, 1984; Schroder, 1985). Although the lung lesions were similar in extent in all three groups, it is possible that the animals in Group III would have recovered from the disease if they were not euthanized at 90 hours post-inoculation, as we did in this study. This scenario is supported by the finding that the number of *M. haemolytica* recovered from the lungs of Group III animals was 100 to 1000 times less than that recovered from the lungs of animals in Group I and II (Table 4). The significantly lower number of bacteria isolated from the lungs of Group III animals is likely due to the presence of relatively larger number of functional phagocytes in the lungs which were protected from Lkt by the peptides. In contrast, the animals in

Groups I and II would have had relatively smaller number of phagocytes in the lungs because of their cytolysis by Lkt.

Molecules such as proteins and peptides are often marginally stable and consequently could be easily damaged or degraded (Tibbetts et al., 2000). *In vivo* degradation when exposed to enzymes results in short biological half-lives (Tibetts, 2000). We believe that prolonging the presence of peptides in the lungs would result in extended protection of the phagocytes from the Lkt, resulting in more effective clearance of bacteria from the lungs which in turn would prevent or mitigate lung lesions. Nanoparticle delivery systems have the potential to improve protein/peptide stability and provide sustained release. Adsorbing the peptides to solid particles such as dextran represents a method of prolonging the presence and activity of the peptides in the lungs. Dextran, under certain controlled conditions, aggregates into porous microspheres, forming crystallized dextran microspheres that can absorb/adsorb peptides, drugs and biologicals, protecting them against degradation and prolonging their release. Such dextran microspheres are available which are biodegradable, biocompatible, non-toxic, non-immunogenic and are removed from the body by normal physiological routes. These characteristics are uniquely advantageous for peptide delivery. Our future experiments will utilize these crystallized dextran microspheres for the delivery of peptides which could be expected to prolong the protective effects of the peptide.

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Table 1: Evaluation and scoring of clinical signs

Clinical signs	Clinical score
Body temperature > 103.5 ^{0}F	2
Inappetance	1
Lethargy / depression	1
Marked weakness / recumbency	2
Moribund state	3
Cough	1
Nasal discharge	1
Respiratory rate > 60 breaths/min	1
Dyspnea	2
Abnormal breath sounds on auscultation	1

Table 2: Bacteria isolated from calves pre-inoculation and at necropsy.

Animal #	Region	*M. haemolytica*		*P. multocida*		*B. trehalosi*	
		Before	After	Before	After	Before	After
16	P	(+)	(+)	(-)	(-)	(-)	(-)
	N	(-)	(+)	(-)	(-)	(-)	(-)
21	P	(+)	(+)	(-)	(-)	(-)	(-)
	N	(-)	(+)	(-)	(-)	(-)	(-)
25	P	(+)	(+)	(-)	(-)	(-)	(-)
	N	(-)	(-)	(-)	(+)	(-)	(-)
26	P	(-)	(-)	(-)	(-)	(-)	(-)
	N	(-)	(-)	(-)	(+)	(-)	(-)
29	P	(+)	(+)	(-)	(-)	(-)	(-)
	N	(+)	(-)	(-)	(+)	(-)	(-)
36	P	(+)	(+)	(-)	Past	(-)	(-)
	N	(-)	(-)	(+)	(+)	(-)	(-)
52	P	(+)	(-)	(+)	(+)	(-)	(+)
	N	(-)	(-)	(-)	(+)	(-)	(-)
68	P	(+)	(-)	(-)	(+)	(-)	(-)
	N	(-)	(-)	(-)	(+)	(-)	(-)
86	P	(+)	(-)	(-)	(-)	(-)	(-)
	N	(-)	(-)	(-)	(+)	(-)	(-)
146	P	(+)	(-)	(-)	(-)	(+)	(-)
	N	(+)	(+)	(-)	(-)	(-)	(-)
168	P	(+)	(+)	(-)	(-)	(+)	(+)
	N	(+)	(+)	(-)	(-)	(-)	(-)
170	P	(-)	(-)	(-)	(-)	(+)	(+)
	N	(+)	(+)	(-)	(+)	(-)	(-)

Before: before the challenge; After: at necropsy; P: Pharynx; N: Nasal cavity; (+): Present; (-): Absent; Past: Pasteurella species

Table 3: Gross pneumonic lesions expressed as a % of total lung volume

Group	Animal #	Age (months)	Lesion (%)	Mean (%)
I	25	4	16.62	10.03 ± 2.85
	16	4	14.03	
	52	6	3.07	
	146	3	8.38	
II	29	4	24.57	12.09± 4.39
	21	4	4.07	
	68	6	10.70	
	168	3	9.01	
III	36	4	15.67	10.53 ± 3.02
	26	4	2.7	
	86	6	13.22	
	170	3	8.51	

Table 4: Number of *M. haemolytica* (CFU per gram of lung tissue) isolated from the lungs of calves at necropsy

	Group I	**Group II**	**Group III**
	2.38×10^6	1.89×10^7	5.29×10^3
	1.50×10^5	6.73×10^5	4.92×10^2
	2.68×10^5	6.50×10^7	5.70×10^4
	2.9×10^7	1.87×10^7	8.00×10^2
Mean	$7.9 \pm 7.0 \times 10^6$	$2.58 \pm 1.374 \times 10^7$	$1.589 \pm 1.374 \times 10^4$

Figure 1:

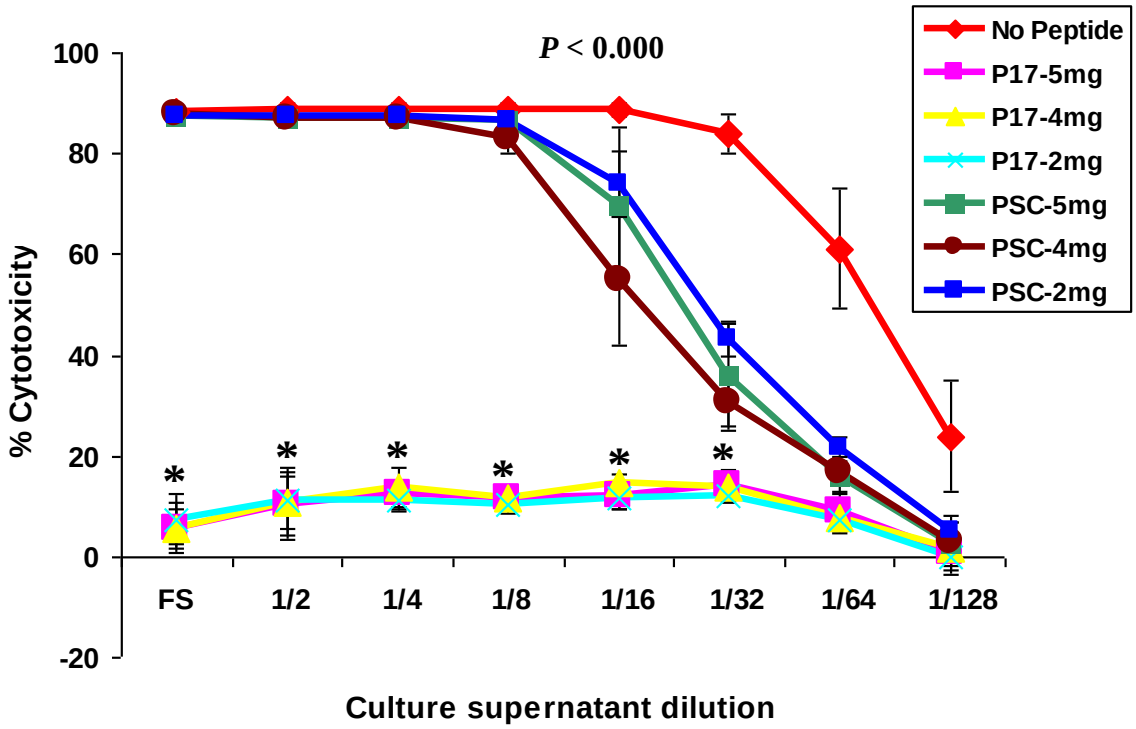

Fig 1: Co-incubation of peptide P17 with *in vitro* cultures of *M. haemolytica* abrogates leukotoxic activity. Supernatant fluids collected from cultures of *M. haemolytica* alone (no peptide), or *M. haemolytica* incubated with the control peptide PSC (PSC-5mg, PSC-4mg, PSC-2mg) or the peptide P17 (P17-5mg, P17-4mg, P17-2mg) were tested for the presence of leukotoxic activity by the MTT dye-reduction cytotoxicity assay at serial dilutions, with BL-3 cells as the target cells. The % cytotoxicity was calculated using the following formula: % Cytotoxicity = [1-(OD of toxin-treated cells/OD of toxin-untreated cells)] X 100. All data are expressed as mean $\pm$ SD (n=3).

Figure 2:

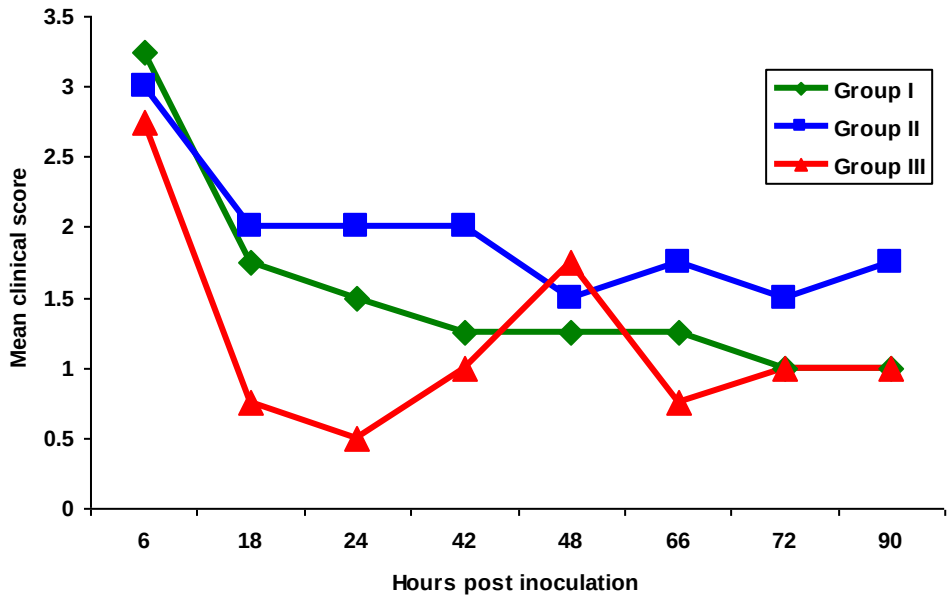

Fig 2: Mean clinical scores of calves inoculated with *M. haemolytica* only (Group I), *M. haemolytica* along with the irrelevant peptide PSC (Group II) and *M. haemolytica* along with the peptide P17 (Group III) at different time points post-inoculation. The mean clinical score of Group III was not statistically significantly lower than that of other groups.

Figure 3:

A **B**

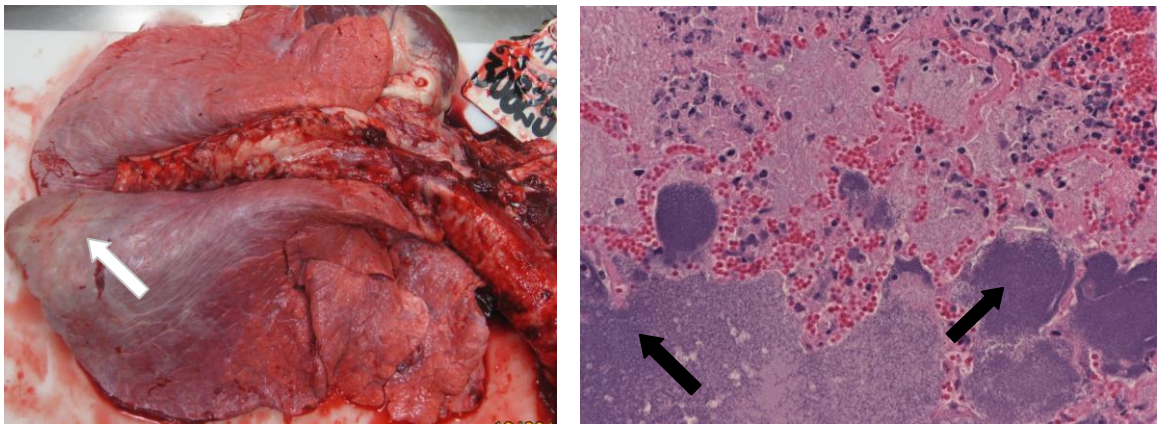

Fig 3: Representative gross- and histo-pathology of the lungs of calves infected with *M. haemolytica* with or without peptides. (A) Gross pathology. Typical gross appearance of the lungs of a calf (#25). The right caudal lobe was consolidated (white arrow) and hard on touch. Fibrin strands on the lung surface indicate pleuritis. (B). Histopathology. Representative lung tissue samples from the affected areas were removed and processed for histopathology as described in material and methods. Alveolar septa are necrotic and the alveoli are filled with streaming mononuclear cells, red blood cells, fibrin and nuclear debris. Large colonies of coccobacilli were present within affected areas of some calves (black arrows). Haemotoxylin and eosin staining and original magnification 100X.

CONCLUSION

Leukotoxin (Lkt) secreted by *Mannheimia haemolytica* is the primary virulence factor; which causes lung injury due to the lysis of alveolar macrophages and PMNs in bovine pneumonic pasteurellosis. Lkt uses CD18, the β subunit of β_2-integrins, as the receptor on ruminant leukocytes. Previously, the Lkt-binding site was mapped to lie between amino acids 1-291 of bovine CD18. In this study, by using a nested set of peptides spanning amino acids 1-291 of bovine CD18, I have mapped the precise Lkt-binding domain to lie between amino acid residues 5 to 17 of CD18 of ruminants. The finding that the Lkt-binding site lies within the amino acids that comprise the signal peptide led to the discovery that the signal peptide of CD18 of ruminants is not cleaved, and that the intact signal peptide of CD18 renders the ruminants susceptible to *M. haemolytica* Lkt. The finding that site-directed mutagenesis of the amino acid glutamine at -5 position to glycine results in the cleavage of the signal peptide and abrogation of Lkt-induced cytolysis of target cells suggests the potential to genetically engineer cattle and other ruminants to contain this mutation. This represents a novel technology to render ruminants resistant to *M. haemolytica* Lkt, and hence less susceptible to pneumonic pasteurellosis and concomitant economic losses.

In the calf challenge study, the total clinical disease scores of calves inoculated with the CD18 peptide analog and *M. haemolytica* were lower than those of calves inoculated with the control peptide and *M. haemolytica* (or *M. haemolytica* alone) but not statistically significant. The difference in percent volume of lungs exhibiting gross pneumonic lesions among the three groups was not statistically significant. However, *M. haemolytica* isolated from the lungs of calves inoculated with the CD18 peptide analog and *M. haemolytica* was 100- to 1000-fold less than those isolated from the calves inoculated with the control peptide and *M. haemolytica* (or *M.*

haemolytica alone). This difference was statistically significant suggesting that the CD18 peptide analog reduced leukotoxic activity in the lungs enabling more effective bacterial clearance by the phagocytes. These findings suggest that prolonging the presence and activity of the CD18 peptide analog in the lungs by using a nanoparticle delivery system such as crystallized dextran microspheres should enhance its protective ability.