Research Summaries

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Antimicrobial Peptides and Surfactant Proteins: Potential New Factors Against Respiratory Tract Infection*

Mark R. Ackermann, *DVM, PhD, Diplomate ACVP*[1]; **Kim A. Brogden,** *PhD*[2]; **P. B. McCray, Jr.,** *MD*[3]

[1]*Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University and* [2]*Respiratory Disease Research Unit, USDA/ARS-National Animal Disease Center; Ames, Iowa 50010;* [3]*Department of Pediatrics, University of Iowa College of Medicine, Iowa City, Iowa*

Abstract

Although some vaccines and antibiotics have been very effective in preventing and treating respiratory disease, they have not been fully satisfactory. Recently, components of the innate immune system have been increasingly appreciated for their role in host defense against microbial pathogens. These molecules include lysozyme and lactoferrin, but recent work in cattle, sheep, man and other species have identified new classes of peptides expressed by respiratory epithelial cells that have potent microcidal activity in nanogram quantities. These peptides, termed antimicrobial peptides (AMP), include defensins, cathelicidins and anionic peptides. Some are expressed continuously whereas expression of others is stimulated by infection/inflammation. In calves, we have found that two AMP from the defensin family, tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP), are expressed in the newborn and increased in response to *Mannheimia (Pasteurella) haemolytica* infection. In contrast, sheep beta defensin-1 (SBD-1) is not induced by infection and, in fact, appears decreased during viral infection with paramyxovirus-3 (PI-3). Decreased SBD-1 by PI-3 may increase the lung's susceptibility to secondary infections or re-infections. Other innate defense molecules include proteins released with lung surfactant. Surfactant protein A and D (SAD) can opsonize and aggregate respiratory syncytial virus (RSV) and activate alveolar macrophages. Preliminary work suggests that chronic bacterial infections result in reduced SpA and SpD expression and we are currently assessing SAD expression in response to PI-3 and RSV. A long-term goal of our work is to identify ways to up-regulate expression of AMP and/or surfactant proteins in the neonate and at times of stress in older animals in order to reduce microbial colonization. Other investigators are seeking ways to utilize AMP's as a new class of antibiotics.

Key words: anionic peptides, antimicrobial peptides, cathelicidins, defensins, epithelia, innate immunity, pneumonia, respiratory tract, shipping fever

Introduction

Respiratory disease is a serious cause of death and economic loss in cattle and sheep.[1,15,25] Of every 1000 cattle that enter feedlots, an average of 12.6 die, and respiratory disease accounts for 57.1% of these deaths and most deaths occur within 45 days of entering the feedlot; a time of great stress.[65] Parainfluenza virus 3 (PI-3) typically causes mild to moderate disease, but may predispose the lung to secondary, more virulent pathogens such as *M. haemolytica*.[1,3,4,28,35] Bovine and ovine respiratory syncytial viruses (RSV) also cause pneumonia and can predispose the lung secondary bacterial infections.[8,27,58,59] The mechanism(s) that lead(s) to increased lung susceptibility to secondary bacterial infection after an initial viral infection is/are poorly defined. Vaccines to paramyxoviruses and *M. haemolytica* can enhance resistance to infection; however these vaccines are not completely effective.[1,2,4,15,19] Despite some improvement in managerial practices, vaccines and clini-

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cal therapies, *M. haemolytica* pneumonia remains a widespread problem and methods to enhance host resistance to colonization and pneumonia by PI-3/BRSV and *M. haemolytica* are needed.

Although the defense of the lung is multifactoral and complicated, it is reasonable to suspect that alterations in the innate immunity of respiratory epithelial cells during and after paramyxoviral pneumonia may have at least a partial role in allowing colonization of secondary bacteria. Recent work by us and others, particularly Dr. Gill Diamond, increasingly indicates that respiratory epithelia of sheep, cattle and other species, produce antimicrobial peptides (AMP), including ß-defensins and anionic peptides, and surfactant protein A and D (SAD) (collectively AMP-SAD), all of which have potent microcidal activity.[13,22,29,36,45,77] A long-term interest of ours is to develop methods to enhance AMP-SAD expression by respiratory epithelia, particularly during the neonatal period and at times of stress/shipping. A better understanding of AMP-SAD expression and the means to enhance their activities is needed because of the high incidence of paramyxoviral and *M. haemolytica* pneumonia in sheep and cattle, the increasing frequency of antibiotic resistance, and the general expectation by consumers for producers to use antibiotics less frequently.

PI-3, RSV and *Mannheimia haemolytica*

Parainfluenza virus type 3 (PI-3). PI-3 infections are often subclinical, and can cause fever, coughing, serous nasal and ocular discharge, and dyspnea. Young and immunosuppressed animals as well as those under stress (overcrowding, shipping) are most susceptible. PI-3 binds glycoconjugates of epithelial cells via activity of the viral hemagglutinin/neuramindase (HA/NA), replicates in epithelial cells of the upper respiratory tract, and spreads to the lower respiratory tract where it has a predilection for bronchiolar epithelial cells.[26,60] Re-infection with PI-3 can occur and secondary infections with bacteria are common. *Mannheimia (Pasteurella) haemolytica, Pasteurella multocida and Haemophilus somnus* are the most common bacteria isolated from calves (and also sheep) infected with PI-3.[1,35,65] In experimental infections of lambs and calves, PI-3 enhances development of lesions when inoculated simultaneously or prior to *M. haemolytica* and leads to decreased alveolar macrophage activity.[28,35,80] Human PI-3 decreases MHC II expression[37] and blocks type I and II interferon expression via altered STAT signaling pathways[88], thereby inhibiting an active (humoral/cell-mediated) immune response, leaving great dependence on innate immunity.

Bovine and ovine respiratory syncytical virus (RSV). RSV is a negative sense single-stranded, enveloped RNA virus of the Paramyxoviridae family.[27,42-44] Transmission occurs from direct inoculation or large aerosol particles into the eye and nose.[42-44] Replication occurs first in nasopharyngeal epithelium followed by replication in the lower respiratory tract. RSV causes hyperplasia, necrosis and sloughing of the respiratory epithelium with increased mucus production and leukocyte infiltration. This can lead to obstruction of small airways and more severe manifestations like tracheobronchitis, bronchiolitis, or pneumonia[8,27,42-44] as well as otitis media. RSV infection is associated with secondary bacterial infections. Major RSV envelope glycoproteins are the F (fusion) and G (attachment) proteins and these induce neutralizing antibodies in the host.[20] However, immunity following infection is not complete nor long-lasting, and re-infection in humans occurs frequently irregardless of age.[43] Traditional therapies (bronchodilators, steroids, ribavirin) in human patients for severe RSV infection generally have no overall significant benefit.[48] A recently introduced recombinant antibody (Palivizumab) to RSV F protein reduced patient hospitalization, but is not yet cost effective for general prophylaxis.

As indicated, RSV is a globally important respiratory disease in cattle, sheep and man. In humans, preterm infants and neonates have elevated risks for severe RSV infection.[43,75] The mechanism(s) for this is (are) not well defined; however, infants, compared to adults, have a diminished capacity for innate and acquired immune responses.[75] Infants have reduced ability for antibody production and this is partially due to the presence of maternal antibodies.[67] Furthermore the increased risk of bacterial infection in infants is due in part to immature neutrophils deficient in antimicrobial peptide expression.[63]

Mannheimia haemolytica. *M. haemolytica* (biotype A) is one of the most important respiratory pathogens of cattle and sheep.[1,4,11,15,25] *M. haemolytica* resides in the tonsil and nasal cavity of healthy ruminants and undergoes selective proliferation during stress or viral infection.[34] It can cause serious outbreaks of acute pneumonia in neonatal, weaned and growing lambs and adult sheep.[25] Neonatal and weaned lambs housed together are particularly susceptible. Live *M. haemolytica* vaccines can enhance resistance to infection; however these vaccines are associated with local tissue reactions, transient anorexia, fever, and even septicemia.[19,34] Experimental vaccines with partially purified *M. haemolytica* leukotoxin can induce an antibody titer,[19] but the effectiveness in preventing pneumonia has not been fully determined. Despite some improvement in managerial practices, vaccines and clinical therapies, *M. haemolytica* pneumonia remains a widespread problem and methods to enhance host resistance to colonization and pneumonia by *M. haemolytica* are needed.

Innate Immunity

The anti-microbial activity of respiratory secretions were first demonstrated by Alexander Fleming.[32,77] He later assessed the anti-bacterial activity of lysozyme, an important innate immune factor in respiratory secretions.[33] Since then, a wide variety of innate immune factors have been identified (Table 1). These factors, working in concert with mucociliary clearance, phagocytosis by alveolar macrophages, enzyme release by neutrophils, and physiologic activity by intravascular macrophages, help to protect the respiratory tract against microbial colonization and accumulation of particulate material. More recently, a group of small antimicrobial peptides and surfactant proteins with potent anti-microbial activity at nanogram concentrations have become increasingly appreciated for their role in innate immunity (Table 1).

Antimicrobial peptides (AMP) of the respiratory tract

AMP are recently characterized small peptides present in epithelial cells, leukocytes and other cells of the body. In respiratory tract secretions, many AMP are constantly and immediately ready for activity against respiratory pathogens. AMP have evolved over the last half a billion years, and are present in a wide variety of living organisms including plants, insects, and vertebrates.[77] The major families of AMP in the respiratory tract of cattle, sheep and man include the alpha, beta and theta defensins, cathelicidins, and anionic peptides. Interestingly, AMP of cattle have been on the forefront of scientific investigation. The first defensin identified was Tracheal Antimicrobial Peptide (TAP), by Dr. Gill Diamond.[29,30,77] Dr. Diamond went on to show that TAP was produced by respiratory epithelia and its expression could be induced by inflammatory stimuli such as IL-1.[74] Shortly thereafter, Lingual Antimicrobial Peptide (LAP) was identified from the tongue of cattle and 14 other defensins were discovered in bovine neutrophils.[77]

Defensins. Defensins are peptides produced by a wide range of species[76] that have activity against bacteria, viral and fungal pathogens.[41,45,76] They are small (roughly 45 amino acids), cationic, and have a diverse primary amino acid structure and a more constant secondary and tertiary structure.[29,77] Defensin families are defined by three to four intramolecular cysteine disulfide bonds. Based on the location of the cysteine moieties and the disulfide bonds, the defensins are further classified into alpha, beta and theta defensins.[6,49] In humans, four beta-defensins (HBD-1, HBD-2, HBD-3, HBD-4) have been described (Table 2), but up to 28 have been predicted. HBD-1 is expressed constitutively while HBD-2, 3, and 4 are inducible.[7,38,39,53] HBD-1 was originally isolated in blood filtrate and is expressed in the lung, skin, gastrointestinal and urogenital epithelium.[55,69,79] HBD-1 has an important role in innate pulmonary defense against pathogens. In patients with cystic fibrosis, high airway salt concentration can render HBD-1 inactive and this may predispose the lung to bacterial infection (40). HBD-1 also participates in cell regulation by promotion of cell differentiation/maturation *in vitro*. HBD-1, HBD-2, and defensins in general, are thought to exert their antimicrobial activity perhaps by forming pores and causing membrane disruption.[36,61] Other activities include healing of epithelium,[68] monocytic, dendritic and T cell chemotaxis,[18,85,87] synergism with other antimicrobial factors such as lysozyme and lactoferrin,[6] and complement activation.[71]

Recent computerized searches of human and mouse chromosomes have found numerous more beta-defensin gene sequences in syntenic clusters.[78] The clusters have similar gene sequences and organization which suggests that each cluster pair arose from a common ancestor that was retained because of conserved function. At least 26 genes in these regions are predicted to be transcribed and, therefore, expressed.[78] Such searches may identify additional beta-defensins of cattle and sheep.

Table 1. Antimicrobial factors in lung surface fluid[77].

Factor/product	Relative concentration	Source
Lysozyme	µg-mg/ml	epithelia, neutrophils
Lactoferrin	µg/ml	epithelia, macrophages
IgA secretory component	µg/ml	epithelia
Phospholipase A2	µg-mg/ml	epithelia
BPI	?	epithelia (?)
Surfactant proteins A, D (SAD)	ng-µg/mll	epithelia
Defensins	ng-µg/ml	epithelia, neutrophils
Cathelicidins	?	neutrophils, epithelia?
Anionic peptide	0.8-1.3 mM	epithelia

? denotes uncertainty

Bovine beta-defensins. Both TAP and LAP are expressed by epithelial cells of the respiratory tract. Their expression can be induced by inflammatory stimuli; however, TAP expression is much greater than LAP.[29,32,74,81] Because of their inductive properties, TAP and LAP are increased shortly after initial infection by certain respiratory pathogens. We have found that LAP expression is markedly increased in calves after infection with M. haemolytica.[81] TAP expression is increased by Gram-negative bacterial components such as lipopolysaccharide and inflammatory cytokines.[29,74] Biologically, the increase of both TAP and LAP may significantly contribute to defense against microbial pathogens shortly after infection and may then allow time for humoral and cell mediated responses.

Sheep beta-defensin-1 (SBD-1) appears not to be induced by infection or inflammatory stimuli (preliminary findings, not shown) and thus has similar constitutive expression and tissue distribution as HBD-1.[51,52] SBD-1 expression is developmentally regulated in late gestation through the neonatal period with maximal expression in some tissues reached weeks after birth.[51] This suggests a window of immature SBD-1 expression in the neonate that provides an environment more conducive to severe PI-3, RSV and *M. haemolytica* infection. We have found that sheep beta-defensin-2 (SBD-2) is also expressed in lung;however, the expression is low.

Anionic peptide was first isolated from respiratory tract of sheep and has potent microcidal activity against a wide variety of pathogens.[12-15] Anionic peptide is a septamer of aspartates, requires zinc as a cofactor for bactericidal activity, and is present in pulmonary surfactant. Much of the work characterizing anionic peptide was done by Dr. Brogden. Briefly, anionic peptide is present in the cytoplasm of individual respiratory epithelial cells of the nasal cavity, trachea, bronchi, bronchioles, and alveoli[12-15] and also in some submucosal gland cells of the trachea of human, ovine and bovine lung. In man, anionic peptide differs in concentration and location of respiratory tract of healthy patients when compared to patients with cystic fibrosis.[14] In sheep, we have determined that anionic peptide production decreases during acute *M. haemoltyica* pneumonia in sheep and cattle (Table 2). We have also determined that during chronic bacterial pneumonia, hyperplastic epithelial cells have increased anionic peptide.[31]

Cathelicidin SMAP29 of ovine origin sheep has very potent activity against respiratory pathogens.[16] Cathelicidins are often expressed by neutrophils[72,90] and cattle, sheep, goats and man expression a number of cathelicidin peptides.[72] Some studies have suggested that some cathelicidins are expressed by respiratory epithelia[29,77] of humans; however, expression in the respiratory tract of cathelicidin in sheep and cattle have not been fully characterized.[72]

Surfactant proteins with antimicrobial activity

Surfactant proteins A & D (SAD) are calcium-dependent lectins and members of the collectin family.[21,24,57,89] SAD expression and function are primarily studied in the lung; however, extrapulmonary expression has been detected elsewhere including the Eustachian tube.[57,70,83] In the lung, SAD are expressed by type-II pneumocytes and Clara cells;[23] however, surfactant protein A has two transcripts, one of which is expressed by submucosal glands of large airways. SAD have an important role in immunomodulation, surfactant homeostasis, and pulmonary defense.[21,22,57] SAD interact with bacterial, fungal and viral pathogens by binding and forming aggregates.[21,47] The SAD aggregates can inactivate the pathogen,[47,84] stimulate phagocytosis,[73] enhance antigen presentation[10] and potentiate oxidant responses of neutrophils,[9] Developmentally, constitutive expression of SAD in the rat increases late in gestation and through the neonatal period.[24] Deficiency of SAD *in vivo* is associated with increased risk for infection,[5] and enhanced inflammation and inflammatory cell recruitment during infection.[62] SAD can reduce RSV infection.[47,48,56,57,62] Severe RSV infection in infants is associated with reduced SAD in the bronchoalveolar lavage (BAL).[56] In mice, SAD inhibit RSV infection *in vivo* and play a major role in clearance from the lungs.[47,57,62] SAD are similar in man and sheep.[22,57,64] We have found that SAD appears to be decreased in bronchioles undergoing hyperplasia during chronic pneumonia (unpublished observations). Such an alteration in SAD expression may allow chronic infections to persist and/or secondary infections to become established in already diseased lungs.

Conclusions

Our long-term goal is to better understand the innate immune system in order to reduce or prevent disease in neonates and in older animals at times of stress (weaning, shipping, overcrowding, inclement weather, etc.). This may be difficult, because it is clear that innate immune factors, including AMP-SAD, are not fully effective in preventing respiratory tract infections in normal animals, since we already know that they express AMP-SAD and infections occur anyway. However, it is equally clear that humoral and cell mediated immune responses are also not fully effective, since animals with normal antibody and cell-mediated responses also acquire infections. Since the time of Jenner and Pasteur, vaccines against microbial pathogens have been developed and refined, yet respiratory disease and pneumonia remain major health problems. Thus, a multi-factorial strategy that includes adequate management, nutrition, housing, ventilation, and optimal activity of all aspects of the immune system, in-

cluding innate, humoral, and cell-mediated responses, appears reasonable. In the meantime, defining and understanding the mechanistic basis of AMP-SAD expression may be useful in making such strategies possible.

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Leptin: The Key to Beef Heifer Puberty and Its Enhancement by Monensin*

Jerome Baker, *MS PhD PAS, Coastal Plain Experiment Station, College of Agriculture and Environmental Science, The University of Georgia, Tifton, Georgia 31793*

Dan T Brown, *MS PhD, College of Agriculture and Environmental Science, The University of Georgia, Blairsville, Georgia*

Mel Pence, *DVM MS PAS, Veterinary Diagnostic and Investigational Laboratory and Department of Large Animal Medicine and Surgery, College of Veterinary Medicine, The University of Georgia, Tifton, Georgia 31793*

Rhonda Vann, *MS PhD PAS, CMREC, Mississippi State University, Raymond, MS 39154*

Duane H Keisler, *PhD, Department of Animal Science, University of Missouri, Columbia, Missouri 65211*

Introduction

Leptin is being investigated as a necessary component to attain reproductive efficiency in beef cattle. The effect of feeding monensin on leptin levels at puberty sheds light on the mechanism of action of leptin. Cows that calve early in the breeding season or have a calving interval of twelve months or less are more profitable than cows with longer calving intervals. For each 21-day heat-cycle a cow is late calving, her calf will weigh about 40 lb less at weaning. Cows that calve early in the breeding season as heifers will be more likely to calve early as cows, and in order for heifers to calve early, they must reach puberty early. The selection and management of replacement heifers influences the reproductive efficiency of the cattle industry by optimizing age at puberty. Heifers that mature sexually at an early age are more likely to settle earlier in a controlled breeding season and wean a heavier calf.

These heifers tend to settle early each breeding season for the rest of their reproductive lives and have heavier weaning weights throughout their lifetime.

With this economic value placed on reproduction, ways to measure, improve and predict reproductive efficiency in cattle are critically important. Age, nutritional status and genetics are factors that control the onset of puberty in beef heifers. Nutritional status of beef heifers and reproduction are intimately related. A target weight of 65% of mature weight at breeding is critical for a successful breeding program. Monensin is an ionophore feed supplement used to increase weight gain and decrease age at puberty in beef and dairy heifers. The mechanism of action to decrease age at puberty has not been fully determined. Pre-pubertal heifers fed monensin produced larger corpora lutea, larger follicles and exhibited increased response to administration of gonadotropins compared to control heifers fed to achieve equal weight gains.