J. Comp. Path. 2014, Vol. 150, 127–137

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INFECTIOUS DISEASE

Hydrogen Peroxide Production and Free Radical-mediated Cell Stress in *Mycoplasma bovis* Pneumonia

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Summary

Mycoplasma bovis causes chronic pneumonia and polyarthritis in feedlot cattle. *M. bovis* infects the lungs of most feedlot cattle, but the majority of calves never develop disease. Competing explanations are that some strains of *M. bovis* are more virulent than others or, alternatively, that calves require some other abnormality to be present in order for *M. bovis* to cause disease. We hypothesize that H_2O_2 production is an important virulence factor of *M. bovis*, causing oxidative injury to lung tissue. A second hypothesis is that isolates associated with caseonecrotic bronchopneumonia have an increased capacity for H_2O_2 production. Immunohistochemical markers of oxidative stress (4-hydroxynonenal, HN) and nitrative stress (3-nitrotyrosine, NT) were compared in lungs of calves with caseonecrotic bronchopneumonia characteristic of *M. bovis* infection, with other forms of bronchopneumonia or with non-inflamed lungs. HN and NT were identified in *M. bovis* pneumonia, mainly in foci of caseous necrosis. HN was not observed in inflamed non-necrotic tissue in lesions typical of pneumonic pasteurellosis. H_2O_2 production by *M. bovis* was identified, but the levels did not differ in isolates from calves with caseonecrotic bronchopneumonia compared with those with non-inflamed lungs or other forms of pneumonia. These findings provide evidence that oxidative and nitrative injury contribute to the formation of the caseonecrotic lesions that are characteristic of *M. bovis* pneumonia and that production of H_2O_2 by *M. bovis* may contribute to this oxidative injury.

Keywords: *Mycoplasma bovis*; nitric oxide; oxidative stress; pneumonia

Introduction

Mycoplasma bovis pneumonia has emerged as a common cause of chronic disease in beef cattle in feedlots and in veal and dairy calves (Caswell and Archambault, 2007). The disease is important because affected calves die or are humanely destroyed because of the costs of treatment, the welfare of chronically diseased cattle and the potential for antimicrobial resistance resulting from chronic therapy with multiple antibiotics. Caseonecrotic bronchopneumonia is the typical lesion of *M. bovis* infection. Histologically, these caseonecrotic foci have loss or absence of alveolar architecture and are filled with the ghost-like remnants of necrotic leucocytes. The periphery of this lesion contains viable macrophages and variable numbers of neutrophils (Gagea *et al.*, 2006a; Hermeyer *et al.*, 2011). *M. bovis* is also detected in other morphological patterns of bronchopneumonia (i.e. acute fibrinosuppurative pneumonia with focal coagulation necrosis and chronic suppurative bronchopneumonia), but its causal role in these lesions is uncertain (Caswell and Archambault, 2007; Hermeyer *et al.*, 2012).

Despite its prevalence and importance, a lack of understanding of how the disease develops has limited the ability to develop effective control measures. Most beef calves become infected with *M. bovis* within a month of entering feedlots (Arcangioli *et al.*, 2008; Castillo-Alcala *et al.*, 2012). It is an enigma that *M.*

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0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2013.07.008

bovis can be isolated from many lungs that are free of pneumonia, yet causes severe and ultimately fatal pneumonia in other cases (Gagea *et al.*, 2006a). One explanation might be that *M. bovis* isolates differ in virulence: some may be commensals, while others may be more capable of causing severe disease. Recent studies have identified considerable genetic diversity in *M. bovis* isolates (Arcangioli *et al.*, 2012; Castillo-Alcala *et al.*, 2012; Pinho *et al.*, 2012; Soehnlen *et al.*, 2011), but no differences in genotype were observed between isolates from diseased versus non-diseased calves (Castillo-Alcala *et al.*, 2012). Alternatively, it may be that all *M. bovis* isolates have similar virulence and that development of *M. bovis* pneumonia is dependent on other factors such as preventative antibiotic treatment. The presence of pneumonic pasteurellosis (Gagea *et al.*, 2006a), or infection with bovine herpesvirus (BHV)-1 (Prysliak *et al.*, 2011) or bovine viral diarrhoea virus (BVDV) (Gagea *et al.*, 2006a; Shahriar *et al.*, 2002) or preventive antibiotic treatment. If the latter is true, then emphasis should be placed on identifying and eliminating the predisposing causes of the disease. In contrast, identification of specific virulence attributes could lead to production of more effective vaccines or diagnostic tests for surveillance and control. Thus, identification of virulence factors and improved understanding of pathogenesis will direct efforts to control this common and economically important disease.

Mycoplasma mycoides subspecies *mycoides* small colony type produces H_2O_2 that causes contact-dependent *in-vitro* cytotoxicity for epithelial cells. Differences between bacterial strains in the levels of H_2O_2 produced correlate with the level of cytotoxicity (Bischof *et al.*, 2008; Pilo *et al.*, 2005). *M. bovis* also produces H_2O_2, in differing amounts by different isolates (Khan *et al.*, 2005). These findings suggest that H_2O_2 production by *M. bovis* could induce lung injury that results in the characteristic caseonecrotic foci and that differences in H_2O_2 production might be an important virulence determinant. Therefore, one aim of the present study was to determine whether the level of H_2O_2 production by different isolates of *M. bovis* correlated with the presence and type of lung lesions in cattle. The second aim was to identify evidence of free radical-mediated injury in different morphological forms of bronchopneumonia, including caseonecrotic bronchopneumonia, acute fibrinous bronchopneumonia with foci of coagulation necrosis and chronic suppurative pneumonia. 4-Hydroxynonenal (HN), a product of lipid peroxidation and a mediator of oxidative stress and apoptotic or necrotic cell death, was used as an indicator of oxidative injury (Uchida, 2003). 3-Nitrotyrosine (NT) was used as a marker of nitrative injury: it is a consequence of peroxynitrite formation in tissues, resulting from the reaction of superoxide anion with nitric oxide or H_2O_2 with nitrite (Sugiura and Ichinose, 2011; Uchida, 2003).

Materials and Methods

Immunohistochemistry (IHC)

The presence and distribution of HN and NT were evaluated by IHC in the lungs of 24 calves. Nine calves had caseonecrotic bronchopneumonia, four had acute fibrinous bronchopneumonia, three had chronic suppurative bronchopneumonia, two had chronic suppurative bronchiolitis and bronchiolitis obliterans and six calves had no lesions of pneumonia. Formalin-fixed and paraffin wax-embedded tissues from a previous study were used and cases were selected based on the prior categorization of histological lesions and pathological diagnoses (Gagea *et al.*, 2006a). Sections stained with haematoxylin and eosin (HE) were reviewed. Lung tissue was sectioned, dewaxed and rehydrated and then rinsed in Tris-buffered saline (TBS). The antigen retrieval, blocking and labelling protocols differed for each antibody.

For detection of HN, sections were treated with proteinase K solution (Dako Canada Inc., Burlington, Canada) for 5 min, washed for 5 min in TBS, incubated in H_2O_2 3% in water for 10 min, washed for 5 min in TBS and then incubated in serum-free protein block (Dako) for 15 min. Primary antibody (Calbiochem, San Diego, California, USA; catalogue number 393206; rabbit polyclonal immunoglobulin [Ig] G anti-HN; diluted 1 in 500 in TBS) was added and slides were incubated at 4°C overnight. For the negative control, slides were processed similarly, but the primary antibody was replaced with negative control rabbit Ig fraction (Dako; catalogue number X0903) at the same protein concentration as the primary antibody.

For detection of NT, slides were submerged in 10 mM citrate buffer pH 6.0 (Dako) at 95°C in a water bath for 20 min, washed with TBS at room temperature for 10 min, incubated in H_2O_2 3% in water for 10 min, rinsed for 5 min in TBS, covered with 1% bovine serum albumin in TBS for 1 h at room temperature, incubated in serum-free protein block (Dako) for 15 min and then covered with primary antibody (Millipore Corp., Billerica, Massachusetts, USA; catalogue number 06-284; rabbit polyclonal IgG anti-NT; diluted 1 in 1,000 with TBA) followed by overnight incubation at 4°C. The negative control was as described above.

The remainder of the procedure was the same for both primary antibodies. Slides were washed five