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Immunolocalization of Pulmonary Intravascular Macrophages, TLR4, TLR9 and IL-8 in Normal and *Pasteurella multocida*-infected Lungs of Water Buffalo (*Bubalus bubalis*)

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Summary

Water buffalo are of considerable economic significance in South East Asia, but these animals suffer from many bacterial respiratory diseases including haemorrhagic septicaemia caused by *Pasteurella multocida*. Bacterial respiratory diseases of animals cause lung inflammation that is characterized by the activation of Toll-like receptors (TLRs) expressed on macrophages, expression of chemokines and recruitment of neutrophils and monocytes. Pulmonary intravascular macrophages (PIMs) present in the alveolar septa play a critical role in lung inflammation, but there are no data on the immunolocalization of PIMs or the expression of TLRs and chemokines such as interleukin (IL)-8, in the lungs of water buffalo. The present study compares the occurrence of PIMs, TLR4, TLR9 and IL-8 in the lungs of normal water buffalo and those infected with *P. multocida*. Labelling of PIMs with the anti-human macrophage antibody (MCA874G) demonstrated an increase in this population in inflamed lungs. TLR4 and IL-8 were detected in the alveolar septa, airway epithelium and endothelium of large blood vessels of normal lungs. TLR9 expression was similar to that of TLR4, but TLR9 was not expressed by the endothelium of arteries and veins. While the expression of TLR9 and IL-8 was increased in the inflamed lungs compared with normal lungs, TLR4 labelling intensity remained unchanged or was reduced. The expression of these molecules potentially plays a role in the regulation of lung inflammation.

Keywords: chemokine; inflammation; pulmonary intravascular macrophage; Toll-like receptor; water buffalo

Introduction

Cattle and water buffalo are a major component of the rural economy in India. These animals provide milk and milk products and also contribute to the economic well being of millions of small-scale farmers. Bacterial respiratory diseases of these species are one of the leading causes of death and economic losses to farmers worldwide (Ribble *et al.*, 1995). The economic losses are due to reduced production, cost of treatments and death of the animals. Although precise economic losses due to respiratory diseases in cattle and buffalo in India are not available, water buffalo suffer from a high incidence of mortality and morbidity caused by *Pasteurella multocida* (Das *et al.*, 2008). Similar pulmonary disease of significant economic impact is caused in North American cattle by *Mannheimia hemolytica* (Lekeux, 2006). These economic losses due to bacterial respiratory diseases in water buffalo continue despite developments in vaccination and therapeutics. The major reason for the lack of progress in developing more effective preventive and treatment strategies against respiratory diseases in cattle and buffaloes is lack of understanding of the respiratory immune system.

Acute lung inflammation induced by bacteria and endotoxins is characterized by the accumulation of neutrophils in the lung (Lentsch and Ward, 2001;

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Hu *et al.*, 2004; Guo and Ward, 2005; Puneet *et al.*, 2005). Innate immune receptors such as Toll-like receptors (TLRs) are the first to recognize the bacteria and their products (Beutler, 2002). TLR4 is an established receptor for bacterial lipopolysaccharide (LPS), while TLR9 binds to bacterial DNA (Beutler, 2002; Murad and Clay, 2009). TLRs, including TLR4 and TLR9, are expressed on a variety of cells, including macrophages and neutrophils, which are central to the recognition and phagocytosis of bacteria. The ligation of TLR4 leads to the activation of macrophages, production of cytokines such as tumour necrosis factor (TNF)-α and chemokines such as interleukin (IL)-8 and subsequent recruitment of inflammatory cells (Andonegui *et al.*, 2003; Janardhan *et al.*, 2006a). Pulmonary intravascular macrophages (PIMs) are an important cell of the respiratory immune system in cattle, sheep, goats and pigs (Warner and Brain, 1990; Staub, 1994). PIMs of cattle and horses express TLR4 and TLR9, phagocytose bacteria and their toxic metabolites and produce inflammatory mediators including cytokines and lipid products (Warner *et al.*, 1987; Singh and Atwal, 1997; Staub *et al.*, 2001; Singh *et al.*, 2004; Singh Suri *et al.*, 2006; Schneberger *et al.*, 2009). Depletion of PIMs attenuates lung inflammation and vascular reactivity in the host species and it is now accepted that PIMs play an important role in the genesis of lung inflammation (Singh *et al.*, 2004; Parbhakar *et al.*, 2005; Singh Suri *et al.*, 2006; Gill *et al.*, 2008; Schneberger *et al.*, 2009).

Recently, the mRNA expression of various TLRs has been demonstrated in many organs including the lung of water buffalo (Vahanan *et al.*, 2008); however, there are no data on the in-situ localization of TLR proteins and the identity of PIMs in these species. The aim of the present study was to demonstrate the presence of PIMs, TLR4, TLR9 and IL-8 in the lungs of normal water buffalo and to compare this expression with that in the lungs of animals with bacterial respiratory disease.

Materials and Methods

Animals and Pathology

The experiments were approved and conducted under the institutional guidelines of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India. Lung samples were collected from normal and *P. multocida*-infected buffalo calves ($n = 4$ each; 4–6 months of age). The inocula used for infection were collected from infected calves that were part of other studies into *P. multocida* infection at the Punjab Veterinary Vaccine Institute. The calves were challenged subcutaneously with an 18 h broth culture of *P. multocida*, which was equivalent to 50×10^6 mouse minimum infective doses as used in potency testing of vaccine candidates. The infected calves died within 40–48 h post-infection. Samples were collected within 1 h of the death from midline areas of the apical, middle and caudal lobes of each lung. These tissues were fixed in 10% neutral buffered formalin for 48 h, processed routinely and embedded in paraffin wax. Sections (5 μm) were placed on poly-L-lysine coated slides and stained with haematoxylin and eosin (HE).

Immunohistochemistry (IHC)

Immunohistochemical protocols are based on those of Singh *et al.* (2004). Lung sections were dewaxed, rehydrated and subject to antigen retrieval by either exposure to pepsin (2 mg/ml in 0.1 N HCl) or microwaving in phosphate buffered saline (PBS; pH 7.2). Following quenching of endogenous peroxidase and blocking for non-specific binding of the antibodies, the sections were incubated with primary antibodies against macrophages, TLR4, TLR9 and IL-8 followed by appropriate secondary antibodies, colour development and counterstaining with methyl green (Table 1). Controls included replacing primary antibody with normal serum or bovine serum albumin or isotype-matched antibodies, and labelling of sections with antibody specific for Factor VIII-related antigen to identify endothelial cells. In preliminary experiments, we found that mouse anti-human CD68 (DAKO Inc., Carpinteria, California), previously shown to react with the bovine pulmonary macrophages, did not label macrophages in the lungs of water buffalo. In contrast, MCA874G, an anti-human macrophage antibody (AbD Serotec, Raleigh, North Carolina), did react with the buffalo lung macrophages.

The total number of cells labelled by the macrophage antibody was counted in each of 10 fields viewed with the ×40 microscope objective in three different sections of lung from each animal. The initial area was randomly selected by moving the microscope stage while not looking through the objective piece. Starting with this field, 10 adjacent fields were examined by moving the microscope stage in a zigzag manner. The mean number (and standard deviation) of cells per field was calculated. Data were compared with the Student's *t*-test.

Results

Histopathology

Lung sections from normal buffalo calves showed normal alveolar septa, airways and large blood vessels