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EXPERIMENTALLY INDUCED DISEASE

Immunohistochemical Labelling of Cytokines in Calves Infected Experimentally with *Mycoplasma bovis*

F. Rodríguez*, P. Castro*, J. B. Poveda†, A. M. Afonso* and A. Fernández*

** Unit of Veterinary Histology and Pathology and † Unit of Epidemiology and Preventive Medicine, Institute for Animal Health, Veterinary School, Universidad de Las Palmas de Gran Canaria, Arucas, Gran Canaria, Spain*

Summary

To gain further insight into the pathogenesis of *Mycoplasma bovis*-associated pneumonia, cytokine expression in different pulmonary compartments was examined. The expression of tumour necrosis factor (TNF)-α, interleukin (IL)-4 and interferon (IFN)-γ was examined immunohistochemically in the lung of 10 calves infected experimentally with *M. bovis*. *M. bovis* antigen was located in respiratory epithelial cells and within inflammatory cells in the airway lumina. Immunolabelling for TNF-α, IL-4 and IFN-γ was usually associated with inflammation, particularly in macrophages and lymphocytes in hyperplastic bronchus-associated lymphoid tissue (BALT), in thickened alveolar septa and in the bronchoalveolar exudate of infected animals. In *M. bovis* infection, macrophage and lymphocyte activation results in expression of a number of cytokines capable of inducing lung lesions and hyperplasia of the BALT. The cytokines examined likely play a role in pulmonary defence against *M. bovis* infection.

Keywords: calf; cytokine; immunohistochemistry; *Mycoplasma bovis*

Mycoplasma bovis is an important pathogen that causes pneumonia and arthritis in calves and young cattle and mastitis in adult cattle (Gourlay *et al.*, 1989; Nicholas and Ayling, 2003). Lesions occurring during *M. bovis* pneumonia include bronchointerstitial inflammation and hyperplasia of the bronchus-associated lymphoid tissue (BALT), collectively known as 'cuffing pneumonia' (Radaelli *et al.*, 2009; Hermeyer *et al.*, 2011). A common feature of infections of the respiratory tract is persistence of the causative organisms at the mucosal surface, which may contribute to the formation of mucosal lymphoid aggregates such as seen in the lungs of animals with mycoplasma infection (Howard *et al.*, 1987).

The local release of cytokines is a key mediator of pulmonary inflammation (Kelley, 1990). Inflammatory cytokines activate the immune system in response to 'danger' and increase the efficiency of an immune response (Murtaugh and Foss, 2002). The induction of cytokines has been suggested to be a pathogenic factor during *M. bovis* infection (Thomas *et al.*, 1991; Jungi *et al.*, 1996; Vanden Bush and Rosenbusch, 2003). The aims of this study were to determine immunohistochemically the expression of cytokines in the lung of calves infected experimentally with *M. bovis* and to investigate the relationship between cytokine expression and the development of pulmonary lesions.

Pneumonic lung tissue was obtained from ten 3-month-old Holstein calves in which *M. bovis* pneumonia had been induced following the protocol described by Rodríguez *et al.* (1996). The animals were given approximately 10^9 colony forming units/ml of *M. bovis* (strain MC1750) grown in *Mycoplasma* broth. Calves were inoculated intratracheally with a 10 ml volume of broth and killed 14 days after inoculation. Lung tissue from three aged-matched calves, inoculated by the same route with 10 ml of sterile *Mycoplasma* broth, served as negative controls. The

Correspondence to: F. Rodríguez (e-mail: frodriguez@dmor.ulpgc.es).

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experiment was carried out in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures (Directive 86/609/EEC).

Lung tissues were processed for histological examination and immunohistochemistry (IHC). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned (4 μm) and stained with haematoxylin and eosin (HE).

For IHC, sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked by incubation of the sections with H_2O_2 0.3% in methanol for 30 min at room temperature. Sections were subjected to heat-induced antigen retrieval (water bath at 98°C) with antigen retrieval solution, pH 6.0 (Dako, Glostrup, Denmark) for 15 min. All tissue sections were incubated with 10% rabbit (for monoclonal antibodies) or swine (for the polyclonal antibody) normal serum (Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature. The primary reagents, each diluted 1 in 300, included mouse anti-bovine tumour necrosis factor (TNF)-α and interleukin (IL)-4 (AbD Serotec, Raleigh, North Carolina, USA) and rabbit anti-bovine interferon (IFN)-γ (MabTech, Nacka, Sweden) antibodies. These were applied overnight at 4°C (Lorenzo *et al.*, 2006). Biotinylated rabbit anti-mouse or swine anti-rabbit IgG (Vector Laboratories), each diluted 1 in 200, were applied as secondary reagents for 30 min at room temperature. An ABC complex (Vector Laboratories) diluted 1 in 50 was applied as the third reagent. The sections were incubated for 3 min with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Missouri, USA) 0.035% in Tris buffered saline containing H_2O_2 0.1%. After rinsing in tap water, slides were lightly counterstained with Harris' haematoxylin and mounted under DPX mountant (BDH Laboratory Supplies, Poole, England). *M. bovis* antigen was immunolabelled as previously described (Rodríguez *et al.*, 1996) by using a monoclonal antibody (MAb 5A10; AFBI Stormont, Belfast, UK) diluted 1 in 5,000. Substitution of the primary antibodies with normal rabbit or swine serum, or monoclonal and polyclonal antibodies specific to other proteins, served as negative controls.

Labelled cells were counted by two pathologists in 20 non-overlapping and consecutively selected high-power fields (0.20 mm^2) from three different sections of lung from each animal. Cells labelled by each antibody were counted in three selected areas corresponding to BALT, alveolar septa and bronchoalveolar epithelium and exudates. Results were expressed as the mean ± standard deviation (SD) number of positive cells/mm^2, and statistical comparison of immunolabelled cells in the control and infected animals was made using the SPSS® programme (SPSS Inc. Headquarters, S. Wacker Drive, Chicago, Illinois, USA).

Pathological findings have been previously reported (Rodríguez *et al.*, 1996). Briefly, well-demarcated areas of consolidation affecting the cranial, middle and accessory lung lobes were observed in all infected animals. By using microbiological and/or immunofluorescence techniques, the participation of viruses, other *Mycoplasma* species or pathogenic bacteria was ruled out in all animals included in the study (Ball *et al.*, 1994).

Microscopically, lesions were characterized by a catarrhal bronchointerstitial pneumonia with varying degrees of peribronchiolar mononuclear cell cuffing (Fig. 1). Accumulations of macrophages and neutrophils in the alveolar spaces and thickening of alveolar septa by mononuclear cells were also prominent features. Immunolabelling of *M. bovis* antigen was demonstrated in the cytoplasm of bronchial, bronchiolar (Fig. 1) and alveolar epithelial cells of lungs from infected animals. Additionally, immunoreaction was prominent in macrophages and, less commonly, in neutrophils within the airways.

Cytokine expression was 'visualized' as brown intracytoplasmic labelling. Labelled cells generally had large oval nuclei and abundant cytoplasm, but sometimes had round nuclei with a narrow rim of cytoplasm. Cytokine expression in infected animals increased significantly compared with the controls in each of the three pulmonary compartments. TNF-α was expressed by macrophages and lymphocytes in the BALT, as well as numerous macrophages

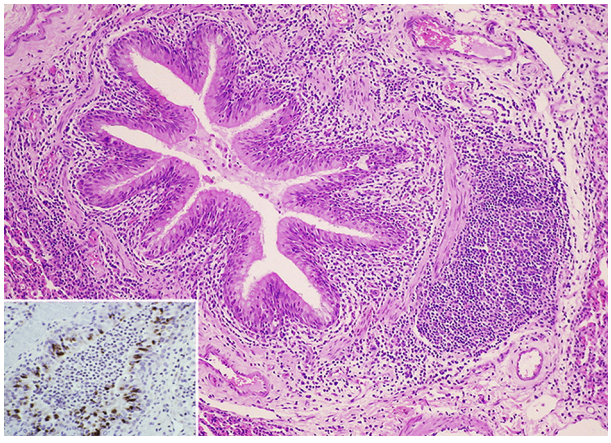

Fig. 1. Lung from infected calf showing peribronchial lymphoplasmacytic infiltration, hyperplasia of the BALT and neutrophils within the epithelium. HE. ×100. Inset: *M. bovis* immunolabelling in the bronchiolar epithelium. IHC. ×200.