J. Comp. Path. 2016, Vol. 155, 130–140

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

www.elsevier.com/locate/jcpa

INFECTIOUS DISEASE

Immunohistochemical Analysis of Leucocyte Subsets in the Sinonasal Mucosa of Cats with Upper Respiratory Tract Aspergillosis

J. L. Whitney*, M. B. Krockenberger*, M. J. Day†, J. A. Beatty*, N. K. Dhand* and V. R. Barrs*

* *University of Sydney, Faculty of Veterinary Science, School of Life and Environmental Sciences, Sydney, Australia and* † *School of Veterinary Sciences, University of Bristol, Langford, North Somerset, UK*

Summary

Leucocyte populations in the sinonasal mucosa of cats with and without upper respiratory tract aspergillosis were compared using immunohistochemistry and computer-aided morphometry. Inflammation was identified in the nasal mucosa of all affected cats, comprising predominantly of lymphoplasmacytic infiltration of the lamina propria associated with epithelial proliferation and degeneration. There was intense and diffuse expression of class II antigens of the major histocompatibility complex, associated with sites of hyphal invasion with hyperplasia and ulceration of the epithelium adjacent to fungal elements. Significantly more $CD79b^+$ cells, total lymphocytes, immunoglobulin (Ig)-expressing cells and $MAC387^+$ cells infiltrated the epithelium and more IgG^+ cells and total Ig-expressing cells infiltrated the lamina propria in affected cats compared with controls. Importantly, the inflammatory profile in affected cats was not consistent with the T helper (Th)1 and Th17 cell-mediated response that confers protective acquired immunity against invasive aspergillosis in dogs and people and in murine models of the infection. This finding may help to explain the development of invasive aspergillosis in systemically immunocompetent cats.

Keywords: aspergillosis; cat; leucocyte subsets; sinonasal immunity

Introduction

Fungal rhinosinusitis has been reported in man, dogs and, with increasing frequency, in cats (Barrs *et al.*, 2012, 2013; Barrs and Talbot, 2014). Feline upper respiratory tract aspergillosis (URTA), which encompasses both sino-orbital aspergillosis (SOA) and sinonasal aspergillosis (SNA), is usually reported in immunocompetent cats. To date, only five of more than 60 cats with URTA have been identified as having concurrent, potentially immunosuppressive diseases including diabetes mellitus in three cats, feline immunodeficiency virus (FIV) infection in one and feline leukaemia virus (FeLV) infection in another (Goodall *et al.*, 1984; Malik *et al.*, 2004; Furrow and Groman, 2009; Barrs *et al.*, 2015; Kano *et al.*, 2015). Recent studies have also detected anti-*Aspergillus* spp. antibodies (of the immunoglobulin [Ig]G class) in the serum of cats with URTA, suggesting appropriate systemic humoral immunity (Barrs *et al.*, 2015).

Although feline URTA appears to affect immunocompetent cats, defects in local defence mechanisms or other immunodeficiencies may permit persistence of infection (Tomsa *et al.*, 2003; Barrs and Talbot, 2014). Purebred brachycephalic cats of Persian lineage are overrepresented in reports of feline SOA (Barrs *et al.*, 2012, 2015). While these cats have clear morphological abnormalities that cause turbulent airflow and mucosal oedema within the

Correspondence to: J. L. Whitney (e-mail: joanna.whitney@sydney.edu.au).

0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2016.08.001

sinuses and nasal cavity (Hendricks, 1992), a defect in innate immunity, which could allow fungal colonization and infection, is also possible (Barrs and Talbot, 2014).

The immune response in invasive aspergillosis (IA) has been most fully elucidated in murine models of the infection and in human patients with pulmonary and disseminated disease (Mirkov *et al.*, 2012; Lass-Florl *et al.*, 2013). The innate immune response to SNA has been described in dogs (Peeters *et al.*, 2005a, 2006, 2007; Mercier *et al.*, 2012; Vanherberghen *et al.*, 2012), but in contrast to cats, which often have invasive fungal rhinosinusitis, this mycosis is non-invasive in dogs (Peeters *et al.*, 2005a). An immune response dominated by the activity of T helper (Th)1 cells, described in dogs with SNA, may prevent dissemination of disease, but is also implicated in the failure to clear localized infection (Peeters *et al.*, 2006).

In order to further understand the immune response to *Aspergillus fumigatus* spp. complex infection in cats, the aim of this study was to describe the infiltrating leucocyte population in the nasal mucosa in cats with URTA.

Materials and Methods

Tissue Samples

Archived sinonasal mucosal biopsy samples from six cats with URTA (two with SNA and four with SOA), obtained endoscopically during diagnostic investigation or at post-mortem examination (sections including nasal cavity and sinuses), were selected for study. The diagnosis of aspergillosis was based on physical examination findings, rhinoscopy, skull computed tomography (CT), positive fungal culture and molecular identification of the infecting isolate by polymerase chain reaction (PCR) (Barrs *et al.*, 2012, 2014).

Signalment, form of disease, infecting fungal species, time of sample collection and treatment history were collected for affected cats. Nasal mucosal biopsy samples were collected from six unaffected control cats with no history or clinical signs of upper respiratory tract (URT) disease undergoing routine post-mortem examination at the University Veterinary Teaching Hospital, Sydney (approved by University of Sydney Animal Ethics Committee, 2014/645). Signalment, underlying disease and previous medications were also recorded for the unaffected control cats.

Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE). Additional sections were mounted on organosilane-coated slides (Silane-Prep Slides, Sigma–Aldrich, St. Louis, Missouri, USA) and stored at 37°C until use.

Histochemistry and Immunohistochemistry

Slides were dewaxed in xylene and rehydrated through graded alcohols to distilled water. Immunohistochemistry (IHC) was performed as described previously (Peeters *et al.*, 2005b; Harley *et al.*, 2011). In brief, slides labelled for expression of IgA, IgG and IgM had endogenous peroxidase activity blocked by incubation in H_2O_2 0.5% in absolute methanol for 30 min. They were then incubated at 37°C in calcium–trypsin solution (pH 7.8) for 1 h to enhance antigen retrieval. Non-specific binding was blocked with rabbit serum in phosphate buffered saline (PBS) for IgG- and IgA-labelled sections and with goat serum in PBS for IgM labelling.

Sections labelled to show CD3 (T-cell marker) and CD79b (B-cell marker) expression were microwaved for 13 min (maximum power) in Tris/EDTA buffer, pH 9.0 (Target Retrieval Solution, pH 9.0, Dako, Glostrup, Denmark), and in pH 6.0 citrate buffer (Target Retrieval Solution, Dako) for sections labelled for class II molecules of the major histocompatibility complex (MHC II, antigen presenting cell and lymphocyte marker). Sections labelled for MAC 387 (myeloid cell marker) expression were incubated in a proteolytic enzyme solution (Proteinase K, Dako) for 15 min. Endogenous peroxidase activity was then blocked by incubation in 3% H_2O_2 for 15 min.

Sections were incubated sequentially with primary and secondary antibody (Table 1) at room temperature. Sections were washed with PBS (for IgA, IgG and IgM labelling) or Tris/EDTA buffer (for CD3, CD79b, MAC 387 and MHC II labelling) between each incubation stage. 3,3'-diaminobenzidine tetrahydrochloride (REAL EnVision Detection System, Dako) was used as the chromogen and sections were counterstained with Mayer's haematoxylin.

Normal feline lymph node was used for positive and negative controls. Negative controls were prepared by substitution of the primary antibody with normal serum from the same animal species used to prepare the primary reagent or Tris–HCI buffer containing stabilizing protein (Antibody Diluent, Dako).

Analysis of Sections

Cellular quantification and assessment of immunohistochemical labelling were performed by JLW using computational morphometric analysis, as described previously (Harley *et al.*, 2011). Sections were