Veterinary Immunology and Immunopathology 166 (2015) 125–131

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Specific faecal antibody responses in sheep infected with *Mycobacterium avium* subspecies *paratuberculosis*

D.J. Begg, K. de Silva, K.M. Plain, A.C. Purdie, N. Dhand, R.J. Whittington*

Farm Animal and Veterinary Public Health, Faculty of Veterinary Science, The University of Sydney, Australia

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 18 June 2015
Accepted 22 June 2015

Keywords:
Mycobacterium avium subspecies *paratuberculosis*
Johne's disease
Sheep
Antibody
Faeces

ABSTRACT

Many studies have examined the serum antibody response to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in cases of Johne's disease (JD), but there are no reports on the mucosal antibody response. Faecal immunoglobulin (Ig) G and IgA ELISA responses were examined from sheep experimentally inoculated with MAP for up to 23 months post inoculation (PI). Corresponding serum IgG responses and the presence of viable MAP shed in faeces were also examined. The sheep were divided into three groups: (i) "un-inoculated controls" ($n = 10$), (ii) "clinical cases" ($n = 8$) which were inoculated animals that developed clinical disease and had moderate to high levels of MAP shedding and (iii) "survivors" ($n = 11$) which were inoculated animals from which MAP could not be cultured from tissues at the conclusion of the trial. Serum IgG responses gradually increased in all inoculated animals, peaking at 12–16 months PI. A significant increase in the levels of MAP-specific faecal IgG and IgA was measured in the survivors at 16 and 17 months PI, while levels in the un-inoculated controls and clinical cases remained at baseline levels. The detection of faecal Ig in the survivors coincided with the removal of sheep that developed clinical disease. The data suggest that some sheep produced MAP-specific IgG and IgA in the intestinal mucosa, which was released into their faeces. We hypothesise that the survivors produced faecal Ig as a direct response to ingestion of MAP associated with environmental contamination from clinical cases. Thus MAP specific mucosal antibodies may play a previously unreported role as part of a protective response triggered by environmental exposure.

1. Introduction

Infection of ruminants with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) can result in a chronic enteritis called Johne's disease (JD). On farm, the disease represents a considerable economic cost to farmers (Bush et al., 2006). The time from infection to appearance of disease may be several years with diagnostic tests not accurate until later periods. Culture of MAP is currently considered to be the most sensitive test for ante mortem detection of MAP infection in sheep, although this technique is time consuming and expensive.

Detection of the host's immune response to MAP is most frequently performed via antibody detection ELISA carried out on serum or milk samples; these assays are high throughput and relatively low cost. However, the sensitivity of MAP-specific ELISAs are generally well below 50%, depend on the stage of disease and vary by animal species and test used (Gumber et al., 2006; Nielsen, 2008). The specificities of MAP ELISAs are high (85–100%), although considered not to be perfect (Nielsen and Toft, 2008).

Most of the commercially available MAP ELISA tests including the IDEXX Pourquier serum antibody ELISA detect the IgG isotype of antibody. Studies have been done to examine other antibody isotypes such as IgG1, IgM and IgA in various ruminant species; the findings indicate that MAP-specific serum antibodies are of the IgG isotype, specifically IgG1 (Abbas and Riemann, 1988; Griffin et al., 2005). Recently, studies on tuberculosis in humans have suggested that using a combination of antigens to detect multiple antibody isotypes may improve the predictive diagnostic outcome (Baumann et al., 2014; Feng et al., 2014).

IgA is thought to play an important role in the immune protection of mucosal surfaces and is the predominant isotype in the intestinal mucosa. In ruminants IgG is transferred into the intestinal mucosa, but is thought to be degraded by proteases which may limit its protective efficacy (Cripps et al., 1974). Interestingly antibody isotypes IgG, IgA and IgM specific to viral infections can be recovered from the faeces of cattle (Heckert et al., 1991; Parreno

* Corresponding author at: University of Sydney, Faculty of Veterinary Science, 425 Werombi Road, Camden 2570, NSW, Australia. Tel.: +61 2 93511619; fax: +61 2 93511618.
E-mail address: richard.whittington@sydney.edu.au (R.J. Whittington).

http://dx.doi.org/10.1016/j.vetimm.2015.06.011
0165-2427/

et al., 2004). Examination of mice indicated that there is a correlation between the concentration of IgA in intestinal lavage fluid compared to a matched faecal sample (Grewal et al., 2000). These findings indicate that a faecal sample from a MAP infected animal might contain immunoglobulins specific for MAP.

Abbas and Riemann (1988) found very little MAP-specific IgA in the serum of MAP infected cattle but these authors did not test faecal samples. There are no published studies in which faeces from MAP-infected animals have been examined for antibodies that may be specific to the infection. In the context of mycobacterial infections, intestinal aspirates, but not serum, of patients with lepromatous leprosy were deficient in IgA (Saha et al., 1978), indicating that a reduction in the amount of mucosal IgA may be associated with disease development.

Secretory Igs play an important role in the intestines, protecting against pathogenic organisms and modulating gut inflammatory homeostasis (Campos-Rodriguez et al., 2013). Examination of faecal IgA and IgG responses may provide information regarding JD pathogenesis, in particular mucosal immunity, throughout the course of the disease. Additionally, the diagnostic potential of detecting these antibody isotypes should be determined. This longitudinal study examined MAP-antigen specific IgG and IgA responses in faecal samples collected from sheep experimentally exposed to MAP compared to unexposed control animals. Faecal samples were also cultured to detect the level of MAP shedding, and matching serum samples were tested for antigen-specific IgG.

2. Methods

2.1. Animals

Thirty Merino wether lambs aged 3 months were sourced from a flock in Armidale, New South Wales (NSW), an area that has no prior history of JD. Absence of MAP infection was confirmed through extensive whole flock faecal tests and serum antibody ELISA (Begg et al., 2010). The animals were moved to a JD – free quarantine farm at the University of Sydney, Camden, NSW and maintained under conventional Australian sheep farming conditions by grazing on open pasture, with unexposed control sheep kept in separate paddocks to the inoculated sheep.

2.2. Ethical considerations

All animal experiments were conducted with the approval of the University of Sydney Animal Ethics Committee.

2.3. Experimental inoculations

The 30 Merino lambs were systematically randomised into two groups. The first 10 sheep were used as the un-inoculated, unexposed controls. The remaining 20 lambs were inoculated orally as described by Begg et al. (2010) with a total of 3.18×10^9 viable MAP strain Telford 9.2, a clonal culture at passage level 5, isolated from sheep faeces and characterised as IS*1311* S strain (Marsh et al., 2006; Marsh and Whittington, 2007).

2.4. Ante-mortem sampling

Faecal samples were collected directly from the rectum and blood samples via jugular venipuncture. Sample collection was performed prior to inoculation with MAP then repeated every 1–3 months post inoculation (PI) until necropsy to monitor the progress of the infection, and the controls were also sampled. Faecal samples were stored at −80 °C until required. Serum from the blood samples was stored at −20 °C until required.

2.5. Identification of clinically diseased sheep

The sheep were culled if they lost more than 10% body weight over a 1-month period. All animals were necropsied as previously described (Begg et al., 2010). Animals culled for weight loss were confirmed to have clinical JD by detection of gross and histopathological lesions consistent with the disease (Perez et al., 1996) and were shown to be infected with MAP as determined by culture of intestinal tissues as described by Begg et al. (2010) and Whittington et al. (1999). Twelve gut tissues, including ileum, jejunum and associated lymph nodes were collected from each sheep for analysis.

2.6. Faecal culture for MAP

Faecal samples were processed and cultured in modified BACTEC 12B medium (Becton Dickinson) containing egg yolk, PANTA-PLUS and mycobactin J as described previously (Whittington et al., 1999). Briefly, after hexadecylpyridinium chloride (HPC), vancomycin, amphotericin B, and nalidixic acid (VAN) decontamination to reduce overgrowth by other bacteria, samples were inoculated into modified BACTEC 12B media and incubated for 12 weeks at 37 °C. Growth index positive samples were confirmed by IS*900* PCR. The weeks to maximal growth (999) were recorded. The weeks to maximal growth can be used as an indicator of the concentration of MAP in the faeces, in that greater numbers of viable MAP in the faeces require fewer weeks to reach maximal growth in culture (Reddacliff et al., 2003).

2.7. Serum antibody ELISA

Serum antibody ELISA (Institute Pourquier (now IDEXX), France) was conducted according to the manufacturer's instructions following the method described previously (Gumber et al., 2006). Results were expressed as signal of the test sample as a proportion of the positive control, corrected for the negative control (S/P%).

2.8. Preparation of faecal samples for ELISA

Faecal samples were removed from the −80 °C freezer and thawed at room temperature. Using aseptic technique, 0.5 g (±0.05 g) of the faecal sample was removed and placed into a 5 mL tube. A 1/10 dilution of the faeces was made by adding 4.5 mL of PBS to each half gram of faeces. The samples were then mixed vigorously on a vortex mixer, incubated at 4 °C overnight and mixed again. A sterile wooden stick was used to break up any clumps in the sample and the sample was mixed vigorously. Using a transfer pipette, 600 μL was then placed in a 1.5 mL tube. The samples then were either frozen at −20 °C until required or processed immediately.

2.9. Faecal IgG and IgA assays

ELISA plates (Nunc Maxisorb) were coated with 50 μL per well of 2.5 μg/mL MAP 316v French pressed antigen (EMAI, NSW Department of Primary Industries) diluted in carbonate buffer (0.1 M, pH 9.6) and stored at 4 °C overnight. The ELISA plates were then machine washed 5 times (Tecan, Austria) using wash buffer (reverse osmosis water with 0.05% v/v Tween 20). The 1/10 diluted faecal samples were thawed if required and centrifuged at 3000 × *g* for 5 min. An aliquot of the supernatant (10 μL) was removed and diluted into 790 μL of PBS from which duplicate 50 μL aliquots were added to wells of the ELISA plate as required. A dilution of 1/800 for the faecal samples was chosen after a set of faecal sample dilutions were tested between 1/60 and 1/16,000. The curves developed indicated that the optimal dilution was 1/800. This dilution gave the best differentiation between faecal samples for both IgA and IgG