A model for the heritability of susceptibility or resilience to Johne’s disease

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Abstract

Resistance and susceptibility to infectious diseases are well-documented in a variety of host species and while many factors contribute to the final outcome of an infection, including environmental modifiers or pathogen virulence, a significant component is the genetic background of the host. Mycobacterium avium subspecies paratuberculosis (Map) can cause a chronic inflammatory bowel disease, Johne’s disease, in ruminant animals such as sheep, cattle and deer. The Disease Research Laboratory (DRL) at the University of Otago has for the past 5 years undertaken a research programme designed to identify immune gene expression markers that characterise individual deer with extreme phenotypes for Susceptibility (S) or Resilience (R) to Johne’s disease using laboratory assays. This became possible after we had identified a number of purebred lines of deer at Peel Forest Estate in South Canterbury which exhibited polarised S or R phenotypes following persistent exposure to high environmental levels of Map. Ex vivo laboratory assay of selected gene expression markers has revealed a panel of targets differentially expressed in deer whose phenotypic status has been confirmed following experimental challenge followed up by necropsy and detailed histopathological analysis.

Introduction

Mycobacterium avium subspecies paratuberculosis (Map) is the causative agent of Johne’s disease (JD), a contagious, chronic granulomatous enteritis that primarily affects the small intestine of ruminants such as sheep, cattle and deer. Outbred populations may exhibit different clinical outcomes to infectious disease and in the context of JD anecdotal evidence from farmers and breed differences seen in cattle (Koets et al. 2000), sheep (Morris et al. 2006, Reddacliff et al. 2005) and deer (Mackintosh et al. 2011) infer that there is a genetic component to JD susceptibility. Given equivalent exposure to environmental stressors and infectious challenge, particular breedlines of farmed red deer exhibit polarised resilience (R) or susceptibility (S) to JD. Phenotypically, S animals develop clinical disease while R animals are comparatively unaffected (Mackintosh et al. 2011), observations that are also supported by anecdotal evidence from deer farmers. The existence of such polarised clinical outcomes allows us to explore mechanistic differences between R and S phenotypes subjected to similar environments, husbandry and exposure to the pathogen. Moreover, is it possible to predict how naive individuals will respond in the face of infectious challenge? In this study we have attempted to model R and S outcomes.
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Based on phenotype following experimental challenge using breedlines of deer which historically have exhibited R and S phenotypes.

In contrast to approaches based purely upon genotype data, other studies have focused upon immune cell readouts assayed from peripheral blood mononuclear cell (PBMC) cultures and observed differences between Map infected PBMC gene expression profiles between clinically diseased animals and uninfected controls (Coussens et al. 2002). The approach adopted by DRL involves the measurement of the expression levels of a series of candidate gene targets (identified both through the literature and as a result of transcriptomics investigations performed previously by DRL) in blood cells from animals with a confirmed S or R genotype to identify biomarkers whose relative expression levels are associated with the S or R phenotype. Our goal has been to identify biomarkers involving different cellular functions in targeted immune cells using the level of specific mRNA produced in peripheral blood cells exposed to Map under laboratory conditions as a proxy for the level of the effector protein molecules produced by the gene. We hypothesised that multiple biomarkers representative of macrophage or T cell function will, when used in combination, provide a predictive diagnostic signature that can distinguish S from R animals. In order to test this hypothesis we have simultaneously monitored multiple markers that influence the function of macrophages and T cells; both of which play an essential role in either the development of disease (S) or protection of the host (R) following Map infection. In experiments over the past 4 years we have screened a panel of >60 individual biomarkers that could be potentially informative in identifying the immune phenotype (function), to differentiate between S and R animals. We have identified markers in S animals that indicate dysfunctional activation of inflammatory cells (macrophages) following exposure to Map under laboratory conditions (Marfell et al. 2013). We have also identified a smaller number of markers that are upregulated in the immune cells of R animals relative to S, following experimental exposure to Map infection. The immune markers that appear to be upregulated in R animals are associated with specific T cells (lymphocytes) that are essential to produce (cell mediated or cellular) protective immunity.

While host breedlines can be determined from stud records/lineage and R/S genotype inferred from this, the host phenotype can only be accurately disclosed following infectious challenge, natural or experimental, whereby susceptible animals are identified through their clinical outcomes and resilient animals by their lack of same. In order to measure differential expression of candidate genes we utilised the JD experimental challenge model described by Mackintosh et al. (Mackintosh et al. 2006) to infect a mob of 39 deer comprising a range of eight different sires with putative R and S phenotypes.

Experimental challenge

In autumn 2013 40 fawns, bred via artificial insemination of random hinds with semen from eight different sires (five progeny per sire) considered likely to be Resilient (n=4) or Susceptible (n=4) based on their known parentage (genotype) and postulated phenotype. Hereafter resilient breedlines are identified as 1, 2, 3 and 4, and Susceptible breedlines as 5, 6, 7 and 8. Weaners were blood sampled twice during March and April to establish baseline gene expression profiles pre-challenge and then transferred to the AgResearch Invermay campus in Mosgiel. In May 2014, 39 deer (one weaner died pre-transport due to misadventure) were experimentally infected orally with an inoculum comprising approximately 4x10^9 live, virulent, bovine field-strain Map bacilli administered over
four days. Map organisms for the challenge trial were recovered from primary tissue homogenates of grossly enlarged mesenteric lymph nodes of a clinically affected deer euthanased the previous spring and archived at -80°C prior to inoculum preparation. Post-inoculation, the challenged animals were run together and managed as a single mob. Animals were monitored and weighed every two weeks to assess overall body condition and analytical samples obtained monthly for Paralisa and gene expression analysis and also for faecal PCR to monitor shedding status. Throughout the course of the experimental challenge animals that failed to thrive and exhibited poor condition or lost 10% of their body weight over a two week period were electively culled; 11 clinically affected animals which met these criteria were euthanased in the period September-December 2014 due to weight loss and loss of muscle mass or scouring compatible with clinical JD. Those animals which were not electively culled due to clinical disease during the challenge trial were necropsied in January 2015 and appropriate tissue samples collected. Photographs were taken, gross signs were recorded and samples of intestines and mesenteric lymph nodes were taken for gene expression studies (snap frozen in liquid nitrogen within 15 minutes of death), fresh for culture/quantitative real-time PCR (qPCR) and fixed in 10% buffered formalin for histopathological examination and lesion severity scoring and subsequent phenotype classification.

Phenotypic scoring

An histopathological grading system devised to score formalin fixed tissue specimens according to tissue damage associated with clinical JD (Clark et al. 2010) was used to assign cumulative phenotypic scores based upon analysis of samples of the gut mucosae and associated lymph nodes (anterior, mid- and posterior jejunum plus associated jejunal lymph nodes as well as ileocaecal valve plus ileocaecal lymph node). Tissue damage contributing to grading scores included evidence of granuloma formation in the mucosa or lymph node containing rafts of acid-fast organisms, caseated areas, lymphoid aggregations, germinal centres, mesenteric granulomas, scattered macrophages in villi and degree of blunting and fusion of villi on the mucosal luminal surface. Histopathological interpretations were performed by a single individual, blinded to details such as genotype or post-mortem findings, in order to maximise scoring objectivity. This grading system assigns a lesion severity score derived from both intestinal and lymph node samples, the sum of which is taken as an overall total lesion severity score which was used to rank individuals according to disease severity and from this to assign a demonstrated R/S JD phenotype. Histopathological phenotypic scoring was used involving a binary system that classified each animal as either R (n=15) or S (n=24). Results obtained for each of the challenged progeny are listed below in Table 1. All subsequent data analysis was based upon these R/S groupings.
Table 1. Description of each of eight different sires, their postulated genotype and demonstrated phenotype based upon histopathological grading of mucosal tissues and associated lymph nodes. R, resilient or S, susceptible. *indicates animals electively culled prior to the end of the trial due to worsening body condition and weight loss.

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Faecal shedding

Faecal shedding of Map bacilli was determined monthly throughout the infection trial using qPCR as described by O’Brien et al. (O’Brien et al. 2011, O’Brien et al. 2013). Prior to challenge Map bacteria were undetectable in the faeces of the subjects whereas immediately post-challenge the inoculum was detectable as pass-through shedding. Although shedding decreased post-challenge it never returned to pre-challenge levels and all challenged animals remained persistent shedders. At the time of peak bacterial shedding in October the S group were shedding on average 100 times more Map bacteria in their faeces than the R group; mean shedding values over time are illustrated in Figure 1 (note that the mean shedding values for the S group are significantly decreased by the elective removal of 11 affected individuals prior to the end of the trial due to clinical JD).

![Faecal Shedding](image)

**Figure 1.** Mean faecal shedding recorded from each of the R and S groups over the course of the infection trial

ELISA results

Peripheral blood samples were assayed for antibody titres raised against two Map antigens, Johnin (PPD-J) and Protoplasmic antigen (PPA) using the Paralisatm test; results are shown in Figure 2. Separation between the R and S groups became apparent four and eight weeks post-challenge for Johnin and PPA antigens, respectively, with the S group exhibiting significantly higher titres of antibody against Map antigen. Note that the mean ELISA values for the S group are significantly decreased by the elective removal of 11 affected individuals prior to the end of the trial due to clinical JD.)
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Laboratory assay for biomarkers associated with R/S phenotypes

In addition to basic measurement of faecal shedding, antibody responses to Map antigens and post-mortem histopathological analysis, a panel of gene expression markers identified in previous studies to be differentially expressed between R and S individuals in similar challenge trials were employed. Our early adoption of a cervine animal model has, of necessity, involved the development of comparatively novel approaches and methodologies with which to interrogate immune responses due to the dearth of experimental tools and reagents with which to study cervine immunology. Monoclonal antibodies are an essential component of conventional immunoassays but there are few well characterised monoclonal antibodies available which are specific for deer. With...
few antibody reagents available to assay for immune markers in the cervine model, gene expression assays were used as they can be designed for almost any gene target for which primary sequence data is available.

The gene expression methodology involves the ex-vivo interrogation of cell types involved with immune function to develop a live cell, bioassay-based approach to immune monitoring based upon transcriptional readouts in response to antigenic stimulation. In previous studies we have focused on ex vivo laboratory culture of monocyte derived macrophages exposed to Map bacilli as a proxy for innate immune responses and reported upregulated expression of a panel of markers in S animals relative to R (Dobson et al. 2013, Marfell et al. 2013). Here we describe a similar approach targeting adaptive immune responses associated with the R phenotype using the peripheral blood mononuclear cell (PBMC) fraction of whole blood stimulated with Staphylococcal enterotoxin B (SEB). SEB is a ‘superantigen’ which acts as a potent polyclonal activator of T-cells to drive transcription of immune response genes. Appropriate T cell responses generated by the adaptive arm of the immune system are responsible for eliciting the protective immunity displayed by individuals of the R phenotype and these responses may be measured through stimulation of T lymphocytes found in the PBMC fraction of whole blood. Lymphocytes are separated and concentrated by density gradient centrifugation and stimulated with SEB to drive transcription of the genes of interest. As T lymphocytes make up the greater fraction of total PBMC cells, a reduction of the sample size down to 20ml still yields sufficient cells to conduct the assay while continuing to disclose statistically significant differential expression of target biomarkers between R and S individuals. Differential gene expression for each of four adaptive immune targets termed Adaptive A, B, C and D are illustrated in Figure 3 below, for three time points during the course of the infection trial (Pre-Challenge, twenty weeks post-challenge and forty weeks post-challenge). Each of these four targets code for key signaling molecules of the mammalian adaptive immune response.
Figure 3. Differential gene expression data for four cytokine markers A, B, C and D measured pre-challenge, twenty weeks post-challenge and forty weeks post-challenge. Data represent the relative quantity of expression of the genes in Resilient and Susceptible animals when stimulated with SEB compared to an unstimulated control. Expression values were normalised to the expression of the Beta-2 Microglobulin gene. Statistical significance determined by using the Mann-Whitney test: *** p<0.0005; **** p<0.00005
Discussion

The JD challenge model in deer has repeatedly demonstrated an ability to produce a spectrum of clinical disease outcomes within a one year timeframe. This would not be achievable in other animal models and provides an efficient model to study differential outcomes following Map infection in animals displaying R and S phenotypes. One component of the challenge model which is difficult to assess or standardise is the overall virulence of the inoculum. The preparation of challenge material from primary, clinically-affected tissue isolates is necessarily crude and quantitative data on the mycobacterial load are only available retrospectively. This makes it difficult to quantify the Map challenge ahead of time or to normalise the infectious Map dose between challenge experiments. Consequently, the infectious challenge and subsequent range of disease outcomes may vary between successive experiments. In the case of the 2014–2015 experiment described here the infectious challenge was at the higher end of the range, resulting in a higher proportion of clinically diseased individuals overall and a greater number of individuals electively culled early due to loss of condition. This is in contrast to a study conducted in 2013–2014 in which the challenge dose and severity of pathology was considerably lower. In this year’s trial, 24/39 (62%) were classified as S phenotype by histopathological grading with the remainder classified as R exhibiting a lesser degree of tissue damage within the mucosal tissue and associated lymph nodes. Sire 1 had a postulated R genotype although 3/5 (60%) of its progeny exhibited an S phenotype with one animal electively culled due to weight loss. Sire 2 also had a postulated R genotype; in this case 4/5 (80%) of its progeny exhibited an R phenotype also. Sire 3 had a postulated R genotype, but only 2/5 (40%) of its progeny were graded as R whereas the remainder were classified as S. Sire 4 with an R genotype produced 75% of progeny with an R phenotype. One of the progeny from each of Sires 1 and 4 was electively culled early due to weight loss. Progeny from sires 5, 6, 7 and 8 proved to be 80% S, 100% S, 40% S and 100% S, respectively. Three out of five progeny from Sire 5 were culled due to progressive weight loss, 3/5 from Sire 6, 2/5 from Sire 7 and 1/5 from Sire 8. The use of a range of selected sires whose progeny have over time been associated with R and S phenotypes allowed the overall outcome of the challenge trial to include polarised pathological outcomes ranging from mild histological tissue damage through to gross visible pathology at necropsy, severe bacterial shedding coupled with high antibody titres. Without such polarised outcomes it would not be possible identify expression markers differentially expressed between the two phenotypic states.

One trend which became apparent during the course of this and also previous longitudinal challenge trials was that the differential expression of particular biomarkers in response either to macrophage (innate) or T cell (adaptive) stimulation observed between R and S phenotypes appears stable over time and do not change significantly as disease progresses. Indeed, pre-challenge responses are strikingly similar to responses observed at 20 and 40 weeks post-challenge suggesting that the observed expression patterns are characteristic of the hosts’ own intrinsic immune response rather than a consequence of infection or disease. If true then this gives reason to expect that a similar immune phenotype could be expected of either naïve or infected individuals. In addition, because the observed differences in T-cell responses between R and S are driven by nonspecific activation of PBMCs by the SEB superantigen, differential expression between the R and S phenotypes is generic and could represent an R phenotype for a number of unrelated microbial infections. Similarly, the differential expression of innate immune gene expression markers observed in in S macrophage cultures, which is indicative of dysfunctional innate (inflammatory) responses, could
reflect a generic defect that could influence an S phenotype’s response to additional microbial infections other than Map. It may well be that the panel of differentially expressed gene expression markers revealed through Map challenge could reveal resilience or susceptibility to other infectious diseases also. While correlation does not imply causation, reproducible differences in an outbred animal model observed across a range of key pathways representing innate and adaptive immunity in R and S phenotypes are strongly suggestive of a real biological phenomenon which may be exploited to gauge immunological competence and to attempt to quantify likelihood of resilience towards infectious challenge.

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