Genetic Resistance to Experimental Infection with Mycobacterium bovis in Red Deer (Cervus elaphus)

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Tuberculosis (Tb) caused by Mycobacterium bovis is a worldwide threat to livestock and humans. One control strategy is to breed livestock that are more resistant to Mycobacterium bovis. In a 3-year heritability study 6 farmed red deer stags were selected from 39 on the basis of their differing responses to experimental challenge via the tonsillar sac with approximately 500 CFU of M. bovis. Two stags remained uninfected, two were moderately affected, and two developed serious spreading Tb. Seventy offspring, bred from these six stags by artificial insemination using stored semen, were similarly challenged with M. bovis. The offspring showed patterns of response to M. bovis challenge similar to those of their sires, providing evidence for a strong genetic basis to resistance to Tb, with an estimated heritability of 0.48 (standard error, 0.096; P < 0.01). This is the first time the heritability of Tb resistance in domestic livestock has been measured. The breeding of selection lines of resistant and susceptible deer will provide an ideal model to study the mechanisms of Tb resistance in a ruminant and could provide an additional strategy for reducing the number and severity of outbreaks of Tb in farmed deer herds. Laboratory studies to identify genetic and immunological markers for resistance to Tb are under way. Preliminary studies showed no associations between NRAMP or DRB genes and resistance to Tb in deer. Patterns of immune responses seen in resistant animals suggest that both innate and acquired pathways of immunity are necessary to produce the resistant phenotype.

Tuberculosis (Tb) is one of the most widespread diseases of mankind and animals. Although the majority of cases of human Tb are caused by Mycobacterium tuberculosis, a small proportion are caused by Mycobacterium bovis carried by cattle and other domestic animals (25). In order to reduce this zoonotic risk, most developed countries have attempted to eradicate bovine Tb from their domestic animals. The problem is not unique to Tb; similar control campaigns have been undertaken for other zoonoses, such as African trypanosomiasis, caused by Trypanosoma brucei (1). In order to control this disease, control of the vector, the tsetse fly Glossina sp., and the maintenance of immunity in the host population are required (13). Over a number of trials involving tickets from various sources, the survival rate of infected tickets was 10% (20). Similarly, when red deer are experimentally challenged by the tonsillar route with 200 to 500 CFU of M. bovis, there is a wide spectrum of disease outcomes (20). Over a number of trials involving tickets from various sources, the survival rate of infected tickets was 10% (20).

We report here the results of a 3-year study that shows that there is usually a range of lesions, although usually less than 10% of the affected animals have severe disease (4, 10). Similarly, when red deer are experimentally challenged by the intratonsil route with 200 to 500 CFU of M. bovis, there is a spectrum of disease outcomes (20). Over a number of trials involving tickets from various sources, the survival rate of infected tickets was 10% (20). Similarly, when red deer are experimentally challenged by the intratonsil route with 200 to 500 CFU of M. bovis, there is a spectrum of disease outcomes (20).

MATERIALS AND METHODS

This study was conducted in three phases. All trials were approved by the AgResearch Invermay Ethics Committee.

Phase 1. Forty-four 2-year-old red deer stags of wide genetic origin and average productivity, in terms of live-weight gains and antler size, were brought from eight commercial deer farms to the Invermay deer farm in late summer (February) 1994. Cell-mediated immune responses (LT) and antibody responses (enzyme-linked immunosorbent assay [ELISA] to M. bovis and Mycobacterium avium tuberculosis [PPD-B and PPD-A, respectively) were measured in a blood test for Tb (BTB), which measures specific reactivity to M. bovis or nonspecific reactivity to other mycobacteria, using a subtractive (PPD-B level minus PPD-A level) assay (13). Semen was collected during the autumn mating season by

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RESULTS

Phase 1. Stags. (i) BTB results. Around 50% of the stags challenged with *M. bovis* produced specific bovine reactivity within 4 weeks, and 30% produced specific bovine antibody within 8 weeks. Cellular reactivity and antibody responsiveness results showed two distinct patterns of immunity that differed markedly between the resistant stags (LSS, 0), which cleared the infectious challenge, and animals which developed tuberculosis lesions (LSS, 1 to 6). Resistant stags developed cellular reactions that were cross-reactive for *M. bovis* and *M. avium* tuberculin, while all infected animals developed specific cellular reactivity to *M. bovis* tuberculin, between 4 and 8 weeks postchallenge.

Patterns of humoral immunity (ELISA) for the resistant and susceptible animals also differed. Resistant stags did not produce antibody to tuberculin at any time postchallenge. By contrast, all susceptible stags (LSS, 6) produced *M. bovis*-specific ELISA reactivity 4 to 8 weeks after challenge. The level of antibody correlated directly with the severity of disease in the infected stags. A summary of lymphocyte transformation and ELISA data for the six selected stags is presented in Table 3.

(ii) Skin test results. The distribution of skin test reactivity for the stags is shown in Fig. 1. Stags with an LSS of 0 had the least skin test reactivity and there was a significant relationship between skin test reactivity and lesion score (y = 8.3/[1 + 1.45] + 0.893/0.424; P < 0.05). There was also a good correlation between the results of the 18-week BTB and the pre-slaughter skin test (Table 3).

(iii) Lesion severity. The numbers of stags classified on the LSS scale of 0 to 6 were 5, 4, 7, 8, 7, 5, and 3, respectively.

(iv) Live weight. There were no significant associations between the live weights of the stags at the time of the challenge

<table>
<thead>
<tr>
<th>Sire stag</th>
<th>Live weight prechallenge (kg)</th>
<th>Sire LSS</th>
<th>Semen quality (% postthaw motility)</th>
<th>No. of inseminations</th>
<th>No. of offspring</th>
<th>No. of offspring with LSS: 0 1 2 3 4 5 6</th>
<th>Mean LSS of offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>406</td>
<td>124</td>
<td>0</td>
<td>65</td>
<td>37</td>
<td>18</td>
<td>2 8 2 5 0 1 0</td>
<td>1.78</td>
</tr>
<tr>
<td>434</td>
<td>101.5</td>
<td>0</td>
<td>60</td>
<td>38</td>
<td>9</td>
<td>0 5 0 3 1 0 0</td>
<td>2.00</td>
</tr>
<tr>
<td>417</td>
<td>117</td>
<td>4</td>
<td>40</td>
<td>38</td>
<td>13</td>
<td>0 8 2 0 1 2 0</td>
<td>2.00</td>
</tr>
<tr>
<td>416</td>
<td>145.5</td>
<td>6</td>
<td>45</td>
<td>40</td>
<td>19</td>
<td>0 7 1 7 3 0 1</td>
<td>2.53</td>
</tr>
<tr>
<td>433</td>
<td>101.5</td>
<td>6</td>
<td>30</td>
<td>31</td>
<td>9</td>
<td>0 0 1 6 0 0 2</td>
<td>3.56</td>
</tr>
</tbody>
</table>

TABLE 2. LSSs and other data for the six selected stags and their offspring
and the subsequent lesion score or the result of the intradermal skin test. Phase 2. Offspring. The parentage of each of the 70 offspring bred by AI was assigned to one of the six stags. They were all negative in a BTB conducted prior to relocation to the quarantined deer farm. Phase 3. Offspring. (i) BTB results. Prior to challenge almost all these deer had high levels of background avian sensitivity. Postchallenge, the patterns of immune reactivity in the offspring were similar to those seen in the stags following challenge, albeit with higher avian reactivity throughout the trial. A summary of LT and ELISA data for offspring in four of the LSS categories (0, 2, 4, and 6) is presented in Table 3. Putatively resistant offspring (LSS, 0) developed or increased their nonspecific cellular reactivity, but not ELISA reactivity, following challenge. Animals that became infected but that did not develop lesions (LSS, 1) produced specific B-cellular reactivity but no ELISA reactions. Specific cellular and ELISA reactivity to M. bovis was seen in the infected offspring with LSSs of 2 to 6, with a correlation between antibody level and disease severity. One LSS 6 animal that had negligible increases in skin thickness at the avian (1.2 mm) and bovine (0.9 mm) sites in the CCT immediately before slaughter nevertheless had high bovine cellular and ELISA reactivities. Another severely affected animal (LSS, 6), which lost weight rapidly and which was euthanized just prior to the scheduled CCT, had high B-cellular and ELISA reactivities just before it died. (ii) Skin test results. The distribution of skin test reactivity to PPD-B for the offspring is shown in Fig. 2. As with the stags, the offspring with a LSS of 0 had the least skin test reactivity and there was a significant relationship between bovine skin test reactivity and LSS for 39 stags challenged with virulent M. bovis. Regression equation, skin thickness = 8.3(+/−1.45) + 0.893(+/−0.424)LSS.

![FIG. 1. Relationship between bovine skin test reactivity and LSS for 39 stags challenged with virulent M. bovis. Regression equation, skin thickness = 8.3(+/−1.45) + 0.893(+/−0.424)LSS.](http://iai.asm.org/)
skin test reactivity and lesion score ($y = 3.389 [+/-0.671] + 0.868 [+/-0.253]$; $P < 0.01$). However, one of the three deer that had a lesion score of 6 had a negligible (<1-mm) increase in double skin thickness, suggesting that it had become anergic to the skin test. A comparison of skin test reactivity to PPD-A and PPD-B sites and the distribution of bovine reactivity are shown in Table 4. There was a high degree of reactivity at the avian site, and half (15 of 29) the LSS 0 and 1 animals had A > B reactivity. Animals with LSSs of 2, 3, 4, and 5 had predominantly B > A activity (33 of 38). By the standard New Zealand interpretation for a positive CCT (increase in skin thickness at the bovine site), both the two LSS 0 animals were negative. By contrast 38% of the LSS 1 and 2 animals and 90% of the LSS 3, 4, and 5 animals were positive, and only one of the two LSS 6 animals was positive.

(iii) Live weights. Parsimonious modeling showed that the sire and the sex of the offspring each had a significant effect on the live weight of the offspring, whereas there was no demonstrable effect on the LSS of the offspring’s live weight at the time of challenge.

(iv) LSSs. The LSSs of the 6 stags and their 70 offspring and the mean LSSs for the offspring of each stag are presented in Table 2. The regression of mean offspring LSS on sire LSS gave a slope of 0.24 corresponding to an estimated heritability for this resistance/susceptibility score of 0.48 with a standard error of 0.096 (95% confidence interval, 0.22 to 0.75; $P = 0.007$). The offspring of infection-free stags had mean lesion scores of 1.78 and 2.0, the offspring of mildly to moderately affected stags had mean lesion scores of 2.0 and 2.53, and offspring of severely affected stags had mean lesion scores of 3.0 and 3.56.

$M.\ bovis$ isolates from typical lesions in both trials had identical restriction endonuclease analysis typing patterns for the challenge organism.

**DISCUSSION**

The results of this trial indicate that there is a strongly heritable basis for the resistance and susceptibility of deer to experimental infection with $M.\ bovis$. The wide range of susceptibility scores in the outbred stags (Table 1) and in the offspring of the selected stags randomly mated with unselected outbred hinds (Table 2) suggests that the general population of deer on farms in New Zealand is likely to be quite heterogeneous for this trait. This is in keeping with the observation that where natural outbreaks of Tb involve widespread infection there is a range of disease severity, with approximately 10% of the animals severely affected. The spread of responses to Tb challenge in the offspring is likely to be due to the fact that the hinds were unselected, and this “diluted” the resistance or susceptibility of the sires and suggests that high Tb resistance or susceptibility may require the homozygous state in a number of genes.

Patterns of immune reactivity seen in animals challenged with virulent $M.\ bovis$ were similar for the stags and their offspring, although there were significantly higher levels of nonspecific sensitization evident in the offspring prior to challenge. This was associated with lower levels of conversion to $M.\ bovis$-specific cellular and antibody reactivities in the offspring, at 8 and 18 weeks postinfection, than in the stags. Although postchallenge cellular sensitization to mycobacterial antigens was seen in all animals within 2 months of challenge, the specificities of these reactions differed markedly. Putatively resistant offspring (LSS, 0), which effectively cleared infection, produced increased levels of nonspecific avian cellular sensitization but no ELISA reactivity following challenge. In stark contrast, susceptible animals, which developed tuberculous lesions, produced specific bovine cellular and antibody reactions. Patterns of reactivity seen in animals challenged experimentally with virulent $M.\ bovis$ were similar to responses seen in naturally infected deer in the field (11). These data highlight the fact that all animals develop acquired immune reactivity following experimental challenge with virulent $M.\ bovis$.

**TABLE 4. Avian (A) and bovine (B) skin test differences, bovine site reactions, and numbers of CCT positives for the 70 offspring**

<table>
<thead>
<tr>
<th>LSS</th>
<th>No.</th>
<th>Difference in reactivity (%)</th>
<th>No. of deer (%)</th>
<th>Double skin thickness at bovine site</th>
<th>No. of deer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A &gt; B</td>
<td>B &gt; A</td>
<td>B ≥ 2 mm</td>
<td>B &lt; 2 ≥ 1 mm</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>27</td>
<td>13 (48)</td>
<td>14 (52)</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>2 (29)</td>
<td>5 (71)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>2 (9)</td>
<td>21 (91)</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1 (20)</td>
<td>4 (80)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0 (0)</td>
<td>3 (100)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>69*</td>
<td>21 (30)</td>
<td>48 (70)</td>
<td>56</td>
<td>8</td>
</tr>
</tbody>
</table>

*One LSS 6 animal was euthanized prior to CCT due to rapid weight loss.*
though at qualitatively and quantitatively different levels. This indicates that the immune response in resistant animals involves acquired as well as innate immunity. The likelihood that protection against certain types of infection appears to require a direct link between innate resistance and acquired immunity has been alluded to by other workers (22). Studies using two direct link between innate resistance and acquired immunity protection against certain types of infection appears to require acquired as well as innate immunity. The likelihood that indicates that the immune response in resistant animals in- volved pathways of immunity. They also suggest that geneti- cally following infection, involves both the innate and ac- quired immunity. They also suggest that geneti- cally susceptible deer may be incapable of developing a pro- tective immune response to *M. bovis* BCG vaccine.

There was a significant correlation between the skin test reactivity and lesion severity for both the stags and the off- spring of the six selected stags. The regression lines had very similar slopes (0.893 and 0.868, respectively), while the y intercept of the line for the young-adult stags (8.3 ± 1.45) was greater than that for the thinner-skinned yearling offspring (3.389 ± 0.671). Thus it appears from these data that delayed-type hypersensitivity responses are better correlated with dis- ease than protection. The exception to this is the animal that appears to have become anergic to the skin test (Fig. 2). It was one of the three deer that had an LSS of 6, and it had a negligible (<1-mm) increase in double skin thickness at the bovine site. Such anergic animals are not unusual in severe outbreaks of Tb in farmed deer and almost invariably test positive for antibody and have severe generalized Tb lesions (11).

The low overall breeding success rate of the AI procedure was primarily due to the light condition of the hinds after a late-summer drought. The actual number of fawns born is not known because the hinds and their offspring were not yarded, identified, and blood sampled for pedigree identification until the fawns were 10 weeks old. Therefore breeding success in this context is a summation of AI success, delivery of full-term fawns, and weaning of the fawns to 3 months of age. The vari- ability in the number of offspring from each stag appears to be largely a feature of semen quality rather than the Tb genotype of the sire. Five of the sires had breeding success rates of 23 to 49%. Only one sire had a particularly poor result (6%), and this animal’s semen had the lowest postthaw motility. There are a number of factors affecting the ability of semen to achieve fertilization, and the only one that is easily assessed is sperm motility (3). The collection of semen by electroejaculation from sedated stags is highly variable, and not all stags give semen of usable quality, especially young stags. It is unfortunate that there were only a total of 11 offspring of susceptible sires because this and the “blunting” of the genetic effect due to random breeding to hinds may have been responsible for the nonparametric distribution of lesion severity (0, 0, 1, 8, 0, 0, and 2 offspring with LSSs of 0 to 6, respectively) in their offspring (Table 2).

This present study is the first time that the heritability of resistance to Tb has been measured in a domestic farm animal. Foundation studies carried out over 60 years ago established that selective breeding produced guinea pigs (32) and rabbits (17) that had increased resistance to Tb. Francis (9) reviewed a number of trials reporting that different strains of rabbits and breeds of cattle showed various degrees of resistance to exper- imental challenge with virulent *M. bovis*. Stead et al. (27) and Houk et al. (15) provided evidence for innate resistance to *M. tuberculosis* in humans. Natural resistance to infection with *M. bovis* BCG has been shown to be partially controlled by a dominant gene in mice, designated formerly as Bcg and now as Nramp (30). The human homologue of mouse Nramp, de- noted NRAMP1, is believed to be associated with susceptibility to leprosy in humans (1). Cattle selected for resistance to an- other intracellular parasite, *Brucella abortus*, also display a deg- ree of resistance to *M. bovis* BCG as demonstrated by the enhanced ability of their macrophages to kill organisms in vitro (26). The association between the bovine NRAMP1 gene and resistance to *B. abortus* and *M. bovis* in cattle is current- ly being investigated (29). A highly informative microsatellite marker derived from the 3’-untranslated region of the cervine NRAMP1 gene (21) showed no allelic associations with either the resistant or susceptible phenotype in the present study (G. Matthews, personal communication). Similarly we have found no association between the DRB genes of the cervine major histocompatibility complex and resistance to Tb (16).

We are currently investigating immunological (12) and ge- netic (6) markers of resistance and susceptibility to *M. bovis* that could be used to identify resistant and susceptible animals without the need to challenge them with virulent *M. bovis*. Such markers could be used to select for resistant animals and to cull susceptible animals. The results of the study reported here suggest that highly susceptible animals are most likely to become infected with *M. bovis*, quickly develop serious Tb, become highly infectious, and possibly become anergic to tuberculin skin tests. It is hypothesized that culling highly sus- ceptible animals and using highly resistant breeding stags will increase the overall resistance of deer herds, reduce the num- ber of new cases, and minimize the risk of serious outbreaks of Tb. This could provide an additional strategy to complement the existing Tb control measures aimed at reducing the number of infected herds.

Genetic and immunological markers for resistance are likely to be similar in all domestic ruminants, and their discovery in deer should provide an additional means of controlling the spread of Tb in all such domestic species. Such markers could be used to screen bulls at artificial breeding centers. The wide- spread use of these resistant bulls could quickly disseminate increased resistance to Tb throughout cattle herds, especially in the dairy industry, where a single bull may sire 200,000 calves in a year by AI. The identification of highly resistant or susceptible animals should also provide us with a new tool to investigate the immune response of deer and other animals to *M. bovis* infection.

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