Vaccination of deer against Tuberculosis; *In vitro* Markers of Immune Protection .

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INTRODUCTION

The tubercle bacillus presents the hosts immune system with the supreme challenge. A slow growing intracellular parasite of low toxicity, it requires a high quality immune response to prevent proliferation and allow effective clearance of infection. M.bovis is recognised as a major pathogen of domesticated deer. Increasing awareness of this fact has led to demands for more effective and less wasteful control programmes to be implemented in New Zealand, North America, Britain and Ireland.

Early work on the pathology of Tb in deer herds established from captured feral animals led to a belief that deer had little resistance to M.bovis . Lesions were characteristically liquefactive and disseminated. Disease spread was often rapid and mortality rates high. The immunocompromising effect of stress may have been a contributing factor to the observed pathology (Griffin 1989b). The patterns of disease have altered markedly after several generations of captivity. Lesions have become more contained as evidenced by granuloma formation, of which an increasing number are calcified. It would appear that adaptation to the farmed environment or selective pressures through death or slaughter of highly susceptible individuals has led to an increasingly resistant gene pool.

While intradermal skin tests can be used to diagnose disease, new laboratory tests such as the blood tests for tuberculosis, BTB (Griffin 1989a; Griffin & Buchan 1989; Griffin & Cross 1989; Griffin, Cross & Buchan 1991), have allowed new precision in determining patterns of immune reactivity in tuberculous animals. Using these new techniques our laboratory has sought to determine if protective immunity to M.bovis exists in deer.

Domesticated deer infected with M.bovis show a spectrum of pathology (Buchan & Griffin 1990). This ranges from multi-focal, disseminated lesions (Generalised Tuberculosis, GTB) associated with extensive bacterial growth to single caseo-calcified lesions. The isolation and calcification of organisms in granulomas as well as the low incidence of Tb (<2%) in most infected herds suggests that deer can develop a protective immune response following exposure to M.bovis (Fig. 1)

Supportive evidence is that uninfected animals maintained for long periods in an infected environment do not necessarily develop infections (Fig. 2).

THE NVL, IMMUNITY TO TUBERCULOSIS OR QUIESCENT INFECTION ?

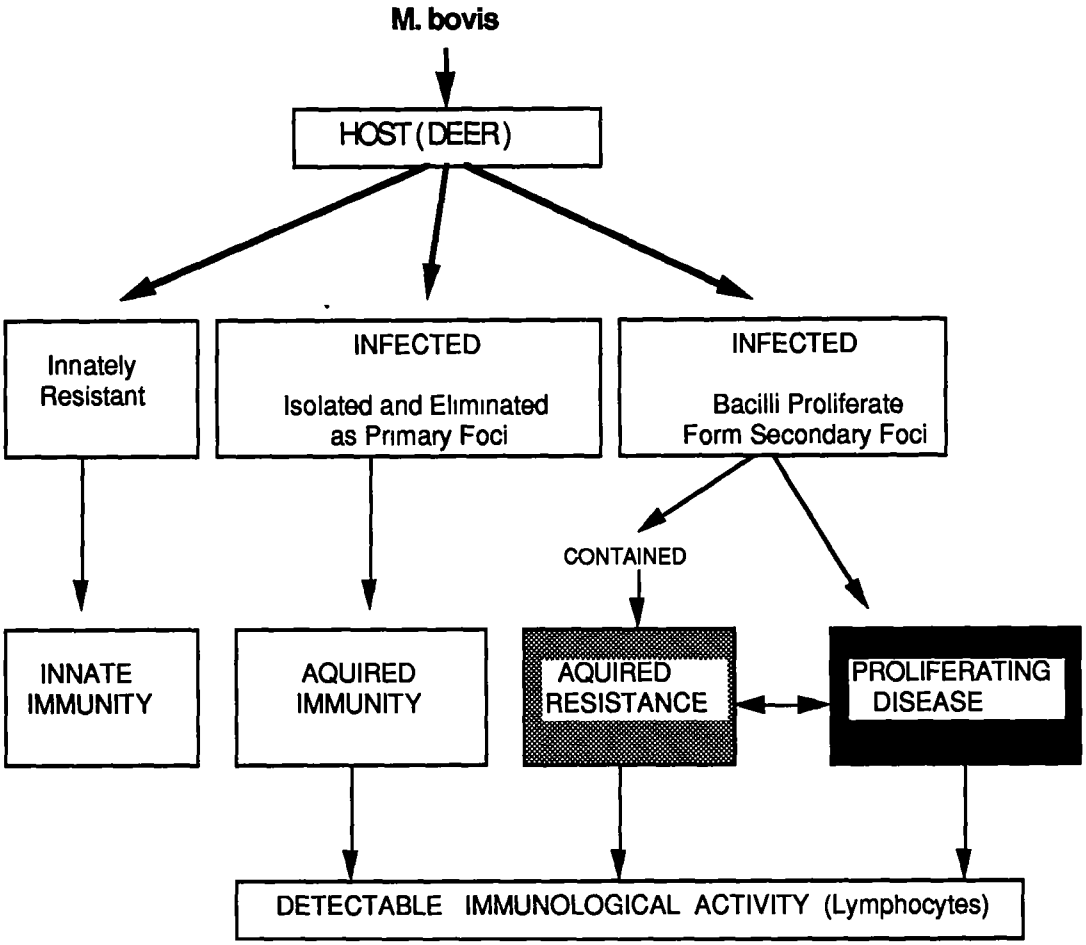

Figure 1. The exposure of an animal to *M.bovis* can have 4 possible outcomes. If disease is contained and eliminated at the site of infection or in the local lymph node acquired immunity will develop and the animal can be considered IMMUNE.

	Change in BTB over 3 Months	
Predicted Risk	**Low - Low**	**High - Low**
No. Tested	65	45
No. Diseased	1	3
% NVL	**98.5**	**93**

Figure 2. Incidence of disease in deer which were exposed to significant levels of disease (26% infection, of which 92% were classified as high reactors to the blood test for tuberculosis ,BTB, at the conclusion of the experiment) for several months . The Low-Low group were classified as at low risk of having Tb lesions at the beginning of the experiment based on BTB data. The High-Low group was composed of animals that changed status during the course of the experiment from showing results consistent with disease at the beginning of the experiment to results consistent with NVL status at the end.

Animals from a herd with significant levels of disease were classified as having a low or high risk of having Tb based on the results of the BTB assays. Low risk animals continued

to maintain resistance to infection even though their lymphocyte reactivity indicated they had been exposed to *M.bovis*. The group that initially showed reactivity consistent with high risk but converted to low risk over the course of the experiment is of some interest. These were found to be predominantly NVL (no visible lesions) at necropsy and may constitute the acquired **resistance** group in figure 1. As such they cannot be considered truly immune as they may harbour dormant organisms capable of causing recrudescent disease.

Throughout the research programme to develop the BTB it was noted that a proportion of animals developed specific lymphocyte reactivity to M.bovis but did not produce specific antibodies. The development of an antibody response to M.bovis is suggestive of a poor disease prognosis as is the development of clinical signs of inflammation (circulating inflammatory proteins such as haptoglobin or fibrinogen). While the presence of lymphocytes sensitive to M.bovis antigens is a good indicator of exposure to tuberculosis, we have found that in the absence of a concomitant antibody response these animals are usually (~80%) free of macroscopic lesions, NVL, at necropsy. This led us to hypothesize that some animals do develop protective immunity to tuberculosis (Griffin, Chinn & Buchan 1988).

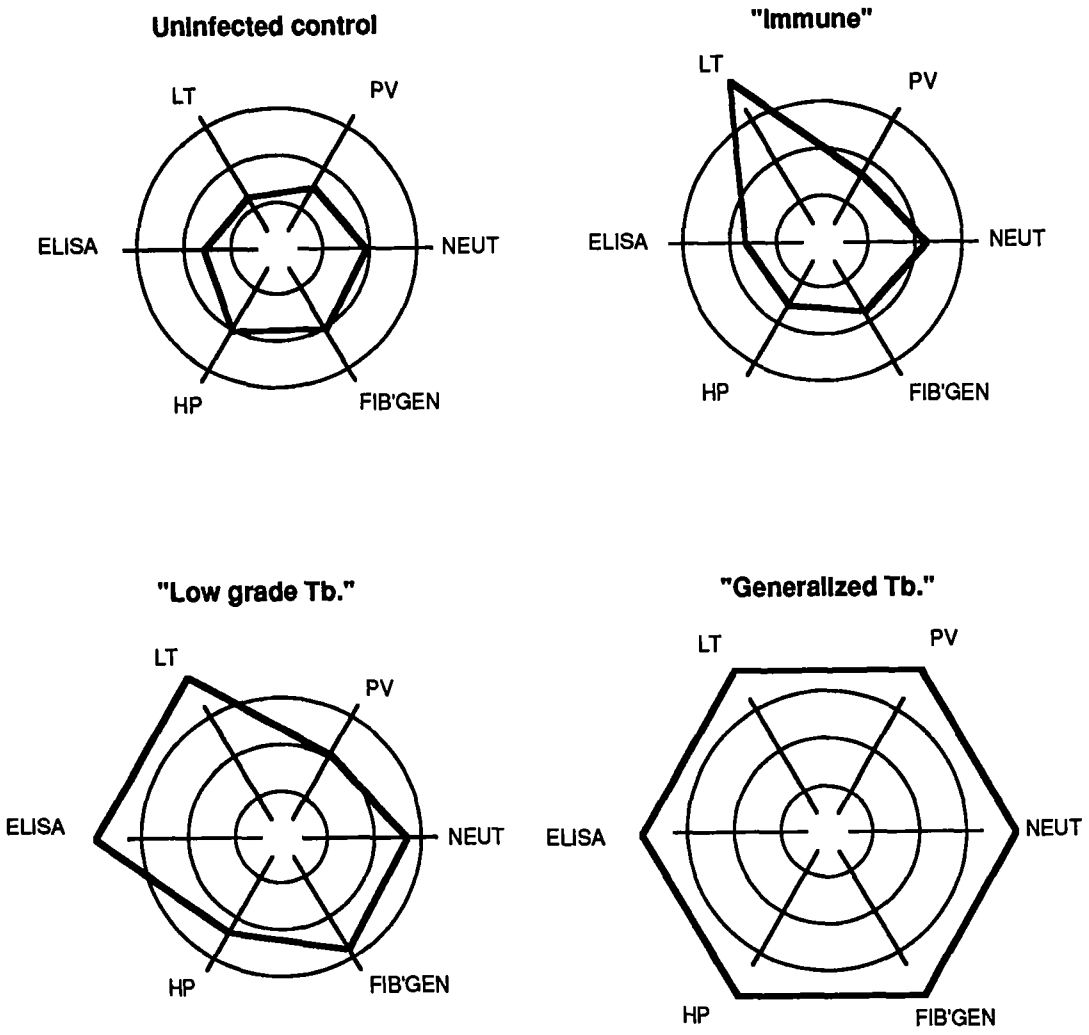

Figure 3. Radial Plots showing typical results of six *in vitro* assays conducted on non-diseased deer, those with 1- several lesions, those with generalised Tb and reactors with no lesions (NVL) at necropsy. The inner circle indicates 2 standard deviations (sd) below the normal range values, the next circle defines the mean value for that assay in deer and the outer circle indicates 2 sd above the normal range of values.

Radial plots of BTB parameters from diseased and NVL animals in infected herds are given in Figure 3. An obvious pattern differentiates lesion bearing animals from those

with no visible lesions (NVL's) at necropsy. While it is possible that some of these NVL's may harbour microscopic lesions, which given time will develop into gross macroscopic lesions, in our experience the majority do not and remain culture negative. Longitudinal studies carried out in this laboratory show that animals which maintain lymphocyte reactivity and do not develop antibody reactivity over a period of many months don't develop macroscopic lesions. While it is not our intention to salvage such animals, because of the danger of undetected disease, it does highlight the concept that protective immunity to Tb occurs and that it may be distinguishable from disease related immune reactivity.

As discussed above good lymphocyte reactivity to M.bovis antigens in the absence of persistent antibody production is one indicator that a protective immune response has developed. It is also known that activation of CD4+ and particularly CD8+ lymphocytes are necessary for a protective response (Kaufmann 1989, Muller *et al* 1987). The activation of CD8+ cytotoxic T-lymphocytes is dependent upon presentation of M.bovis antigens on the surface of Antigen Presenting Cells (APCs eg. macrophages) by special proteins (MHC Class I proteins). The activation of CD4+ helper/regulator T-lymphocytes is dependent upon presentation of these antigens on APCs by another group of proteins (MHC Class II proteins). By using monoclonal antibodies against these proteins *in vitro* and measuring the degree to which they inhibit lymphocyte reactivity to bovine purified protein derivative (PPD), we have been able to determine differences in the degree to which the two lymphocyte subpopulations are activated in diseased and non-diseased (Immune?) animals *in vivo* (Fig 4).

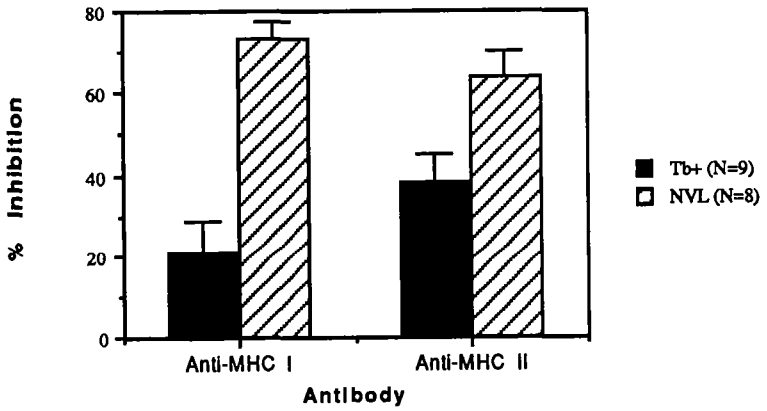

Figure 4. The ability of antibodies to MHC proteins to inhibit *in vitro* lymphocyte transformation responses to bovine PPD in deer with tuberculosis and reactor animals found to have NVLs at necropsy. Anti-MHC I inhibition suggests inhibition of CD8+ lymphocytes whereas anti-MHC II inhibition suggests inhibition of CD4+ lymphocytes.

Analysis of lymphocyte reaction patterns to bovine PPD suggest that MHC class I reactive cells (CD8+ lymphocytes) are particularly important in the protective response to Tb in deer. Unequivocal evidence that these animals are immune awaits more detailed investigation of the Cervine immune response to M.bovis.

VACCINES FOR TUBERCULOSIS

Currently studies are underway in our laboratory which are attempting to induce protective immunity to Tb by vaccination with various preparations of BCG (bacillus Calmette-Guerin). Vaccination offers the most cost effective and efficient answer to disease spread in any large host. The question of whether a danger is associated with using a live, though attenuated vaccine, in deer is still a major concern. It must first be shown that the vaccine is incapable of causing spreading infection in deer.

IMMUNISATION SCHEDULE FOR VACCINE TRIAL

	Organism	Dose (No./Animal)	Adjuvant	Route
Group 1	Live BCG	$5x10^6$	NIL	Subcut.
Group 2	Live BCG	$5x10^6$	STM	Subcut
Group 3	Live BCG + Killed M.vaccae	$5x10^6$ + $10x10^6$	NIL	Subcut
Group 4	Killed BCG	$5x10^6$	STM	Subcut
Group 5	NIL	NIL	STM	Subcut

Figure 5. Vaccination Protocol. STM (Mineral oil containing Marcol, Tween and Arlacel).

To this end 25 deer were divided into 5 groups and given various vaccines subcutaneously in the cervical region. The experimental procedure is given in Figure 5. The lymphocyte transformation, antibody and inflammatory protein responses as well as phenotype analysis of lymphocyte subpopulations were assayed each week. The animals were boosted with the same regime at 8 weeks and analysed for a further 12 weeks prior to necropsy. Detailed necropsy showed no gross lymph node pathology in any of the animals. The ipsilateral prescapular (draining node) showed enlargement in some animals. None of these nodes displayed any macroscopic signs of infection. The site of the inoculation showed no pathology apart from some fibrosis and small focal lesions 20% of the animals that had received vaccine in oil. Pooled nodes from the head, thorax and abdomen as well as tissue from the innoculation site were cultured and found to be negative except in one animal from group 3 (live BCG + killed *M vaccae*) where a single organism was cultured from the prescapular node draining the injection site.

The experiment has shown that

1. Deer are capable of containing and eliminating live BCG. Ongoing experiments suggests vaccinated animals pose no danger to in-contact animals as there is no evidence of sensitisation of control (non-vaccinated animals) during the course of the trial.
2. The vaccination procedure need not pose ethical problems in respect to unacceptable abscessation at the site of inoculation or in draining lymph nodes.
3. Small doses of organisms invoke an immune response in deer. In mice 10^8 organisms are commonly used whereas $<10^7$ organisms gave significant levels of transformation and antibody production in deer.

While all animals converted to become skin test positive by the conclusion of the experiment, evidence from *in vitro* assays suggest that vaccine induced immunity can be differentiated from disease related immune reactivity. Vaccinated animals produced no antibody to a 23kD antigen of M.bovis (Figure 6) whereas at least 70% of diseased animals produce antibody to this antigen (Griffin & Buchan 1991).

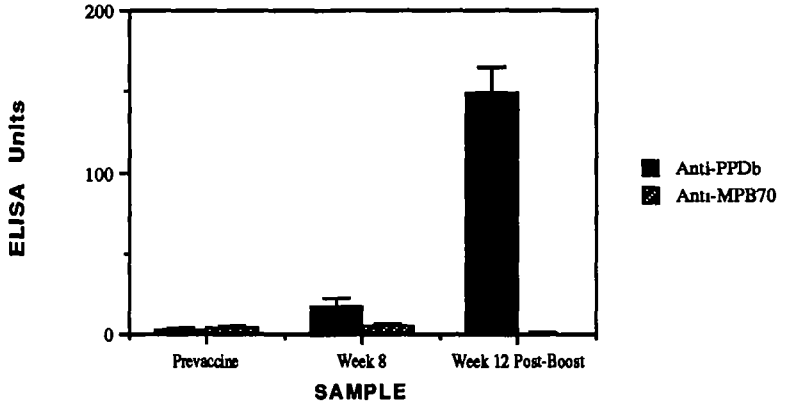

Figure 6. The mean antibody levels to bovine PPD and MPB70, found in 5 deer vaccinated with live BCG in oil. Levels were measured before vaccination, 8 weeks after vaccination and then 12 weeks after a secondary boost of live BCG in oil.

This suggests the possibility of identifying diseased animals by the presence of immune reactivity to certain antigens absent from BCG.

Given that M.bovis is found in feral animals within New Zealand the control and eradication of tuberculosis from deer and cattle herds in Tb endemic areas is unlikely to be achieved without the development of a 'protective' vaccine.
In vitro studies have shown that certain immune parameters are associated with the ability to resist infection by M.bovis. The ability to detect differences between **immune** and **diseased** deer will be an important consideration in vaccine development.

We have shown that deer do produce significant levels of immune reactivity to Tb vaccines and that live attenuated vaccines based on BCG do not pose a health risk. Currently we are investigating strategies to optimise vaccine delivery, and are evaluating alternatives to BCG as candidate vaccines for Tb in deer.

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