Chapter 1. Introduction and literature review

Introduction

Johne's disease is a chronic enteropathy of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*). First described in European cattle in 1895, the disease now causes significant economic losses worldwide. This review concentrates on the pathogenesis of Johne's disease in ruminants, and in sheep in particular. Much of the early experimental work in sheep was carried out as a model for the bovine disease and overall much more work has been done on the disease in cattle so considerable reference to bovine studies will be made. Other aspects of the biology of *M. a. paratuberculosis* infection will be discussed briefly, and in more detail where they have particular bearing on the understanding of pathogenesis.

Ovine Johne's disease (OJD) in Australia is an emerging disease. From its first diagnosis in the central tablelands of NSW in 1980, the disease has spread to involve 500 flocks across NSW, with almost 2000 more under suspicion by mid 2000. Infection is also present in South Australia, Tasmania and Victoria, but has not been reported from the other states. Control programs are necessary to protect sheep populations free of the disease and to reduce economic impacts, including not only direct production losses due to disease, but also those due to trade restrictions. To address these concerns and to determine whether eradication of OJD from Australia was feasible the National Ovine Johne's Control and Evaluation Program (NOJD) was established in 1998. Further research into OJD, especially in the Australian context was identified as a priority. It is against this background that this PhD program was undertaken.

One control option being examined by the NOJD is destocking of an infected property, allowing pastures to lose infectivity over time (currently deemed to be two consecutive summers) then restocking with uninfected sheep. The ultimate test for pasture infectivity is whether sheep exposed to the pasture subsequently show evidence of *M. a. paratuberculosis* infection. Current tests for infection are unreliable in the early stages of the disease so it may be many years before the efficacy of a destocking program is known. Better tests for early infection would allow timely assessment of pasture infectivity. But improved tests and confidence will only come with increased understanding of the pathogenesis of the disease. Much of the experimental work on OJD has been done using isolates of bovine strains of *M. a. paratuberculosis*. Work with the more host-adapted ovine strain, especially Australian isolates, in
Australian sheep under Australian conditions is necessary if research results are to be confidently applied in local control programs. Also, the status of the “uninfected” sheep used for restocking is difficult to assess with current tests. Uninfected animals for restocking are currently made available through market assurance schemes, in which repeated flock testing gives increased levels of confidence that the sheep are free of *M. a. paratuberculosis* infection. An alternative strategy, where destocking is not an option, is to manage the flock to reduce the impact of the disease, and one possible approach is the use of resistant sheep.

The research described in Chapter 2 of this thesis deals with the development of techniques for the enumeration of Australian ovine isolates of *M. a. paratuberculosis*, which will facilitate further research into both the pathogenesis and epidemiology of OJD. Chapters 3 and 4 address the early detection of infection in lambs exposed to Australian ovine strains of *M. a. paratuberculosis*, in a controlled laboratory pen trial and then in the field. Examination of sheep at this early stage of infection, in particular the use of culture from tissues, allowed assessment of the current diagnostic techniques for detection of early infection in sheep and some investigation of early immune responses in Chapter 5. Finally, possible associations with host susceptibility to Johne's disease of polymorphisms in a number of genes associated with immune function were examined in Chapter 6.

**Aetiology**

*M. a. paratuberculosis* is a facultatively anaerobic, acid fast, partially gram-positive bacterium, a member of the *Mycobacterium avium* complex. In common with all mycobacteria it has a thick lipid-rich cell wall which is responsible not only for its acid fast staining but also for its resistance in the external environment and for some of its ability to survive within the macrophages of infected animals.\(^{136}\) *M. a. paratuberculosis* is traditionally distinguished from the ubiquitous *M. avium* by its extremely slow growth in culture and its requirement for exogenous mycobactin for *in vitro* growth. Mycobactin is a high molecular weight complex lipid that chelates iron for storage in the bacterial cell wall.\(^{67}\) Mycobactins are unique to mycobacteria and nocardiae, but absent (or present in very low levels) from most strains of *M. a. paratuberculosis* and some strains of *M. avium*. Recently molecular techniques have identified multiple copies in the genome of a DNA insertion sequence, IS\(900\), which is considered unique to *M. a. paratuberculosis*, although very similar insertion sequences are sometimes present in other as yet uncharacterised mycobacteria.\(^{77,345}\) *M. a. paratuberculosis* is considered to be an obligate pathogen of mammals, depending on its ability to survive and multiply intracellularly.\(^{336}\) Nevertheless it has considerable ability to survive in the external environment. Specific studies using bovine strains have shown survival times of more than 6 months in slurry at 5 °C,\(^{148}\) 8 months in faeces exposed to ambient conditions,\(^{196}\) and 19 months in water at a constant 38 °C.\(^{183}\) Of interest
also is the demonstration that organisms may survive in cultures under conditions of continuous desiccation for at least 47 months. The organism is sensitive to UV radiation, and does not survive well in contact with urine. In the only study on ovine strains of *M. a. paratuberculosis* survival was demonstrated for 13 months on shaded pasture plots, and for 7 months in exposed situations. Preliminary observations also indicated that survival may be better in water (such as in troughs or farm dams) than on adjacent pasture, and the possibility that it may survive and even multiply within aquatic invertebrates was considered.

**Host and geographic range**

Johne's disease is primarily a disease of ruminants. It has been recognised as a problem in cattle for more than a century and in sheep and goats since 1916. It is present on all continents (except Antarctica?) and spreading insidiously. Prevalence estimates of 15% and 18% of cattle in UK and USA respectively have been documented and economic losses in the billions of dollars suggested. In Australia it was first diagnosed in cattle in 1925, goats in 1977 and sheep in 1980. There is some evidence that disease is more prevalent in areas with acid soils, for example in sheep in South Africa and in cattle in Wisconsin. These associations were independent of stock density and have been suggested to be due to increased availability of iron. There is some experimental support for this contention in that numbers of *M. a. paratuberculosis* in granulomas of experimentally infected mice are correlated with iron intake. However, in cattle dietary iron levels had no effect on the development or severity of experimental Johne's disease.

As well as sheep, cattle and goats, Johne's disease has been described in many species of deer, moose, antelopes, and numerous wild relatives of the sheep and goats. (various authors cited by Chiodini et al) It also occurs in the camellids, and has affected alpacas in Australia. Infections in wild ruminants are potential reservoirs of infection for domestic stock. Other non-ruminant livestock species are also potentially susceptible as shown by experimental infections. Using very large doses pigs were infected (showing typical lesions and excreting the organism in faeces) by both oral and intravenous (IV) routes, but such infection could only be produced in horses by the IV route. There are no good reports of natural infection in these species.

Recent studies have demonstrated infections in a wide variety of non-ruminant wildlife species in areas in Scotland where paratuberculosis in domestic ruminants is prevalent. Severe infections with advanced histopathological lesions and faecal excretion were present in 22% of
wild rabbits examined. M. a. paratuberculosis was isolated from fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare, and badger, with histological lesions present in fox, stoat, weasel, crow and wood mouse. The lesions in the carnivores were not as severe as those seen in ruminants or wild rabbits. Other non-ruminant species with reported natural M. a. paratuberculosis infections include a rhinoceros, stumptail macaques and kangaroos (RW personal communication).

In the search for a suitable small animal model for Johne’s disease there have been many attempts to infect a variety of monogastric laboratory animals. Lominski et al cite some 35 references to studies prior to 1950, where investigators failed to infect one or more of cats, guinea pigs, hamsters, mice, rabbits, rats, chickens and pigeons, or produced only lesions or reactions associated with the injection of foreign material. In a series of experiments in the 1970’s Larsen et al successfully infected lemmings orally but found Mongolian gerbils and chickens to be more resistant. Subsequent investigators have succeeded in establishing infection in monogastrics but only certain strains of mice (particularly immunodeficient mice), hamsters and neonatal rabbits are reliably infected orally. Chickens immunosuppressed by cyclophosphamide and concurrent infection with Infectious Bursal Disease Virus were susceptible, developed typical intestinal lesions and excreted the organism in faeces. In most laboratory animals very large doses (in excess of $10^8$ organisms) are required to establish infection, although severe combined immunodeficient beige (SCID bg) mice could be infected orally with as few as about $10^5$ organisms. Parenteral infection routes (intraperitoneal or IV) have been used in the past for successful infection of laboratory animals. While of limited use in pathogenesis studies it is of interest to note that in almost all of the above studies, regardless of the route of infection, infection localised in intestine and mesenteric nodes. The fact that most laboratory species seem inherently more resistant to infection than ruminants, means that pathogenesis studies of M. a. paratuberculosis infection in laboratory animals need to be interpreted with caution.

The clinical and pathological similarities between ruminant paratuberculosis and Crohn’s disease in humans have been recognised for nearly a century. A recent paper specifically reviews evidence for the role of M. a. paratuberculosis in Crohn’s disease. Only in recent years have technical advances (improved culture methods, polymerase chain reaction (PCR), new antibiotics) provided evidence that M. a. paratuberculosis may indeed be a cause in some cases. This evidence includes culture of the organism or detection of cell wall-defective forms from
affected bowel, response to treatment with antimycobacterial antibiotics and the detection of humoral immune responses. Moreover the occurrence of severe clinical paratuberculosis in a colony of stump-tail macaques housed at a research institution demonstrates unequivocally the potential susceptibility of primates to infection with *M. a. paratuberculosis*. On the other hand, another recent review concentrates on the environmental factors associated with Crohn’s disease in man and concludes that the aetiology is multifactorial and that most studies suggesting a potential pathogenetic role of *M. a. paratuberculosis* in Crohn's disease could not be reproduced by others. They cite convincing data from genetic animal models and twin studies which indicate that environmental factors and the intestinal bacterial flora interact with susceptible hosts to cause the inflammatory bowel diseases. But these conclusions do not exclude a role for *M. a. paratuberculosis* as a contributing aetiology in some cases of Crohn’s disease, and transmission to man is a risk that cannot be ignored. The potential for widespread human exposure to *M. a. paratuberculosis* is considerable, given the prevalence of the disease in dairy cattle in many countries, the extensive marketing of fresh milk, and the fact that pasteurisation, whilst greatly reducing numbers, may not entirely eliminate viable *M. a. paratuberculosis* from milk. Possibly of greater concern is the use of unpasteurised milk from cattle, goats or sheep in the alternative “health food” market. And whether or not there is a significant risk to human health, there is a perceived risk which adds to any economic incentives for the control of paratuberculosis in the livestock industries in many countries.

**Epidemiology**

**Transmission of infection between animals**

**Faecal excretion and oral infection:**

Faecal excretion is the main source of environmental contamination with *M. a. paratuberculosis*, and is probably the main means of transfer of infection between animals. Exposure to organisms originating in faeces can occur by ingestion of contaminated pasture, soil, or water, or from faeces on the teats. The recovery of *M. a. paratuberculosis* from trichostrongylid larvae cultured in infected faeces suggests indirect transmission via helminth parasites might also be possible.

Clinically affected sheep or cattle can excrete enormous numbers of organisms in their faeces. Sheep infected with the multibacillary form of the disease were shown to excrete $10^8$ organisms per gram of faeces, measured by end-point titration in Bactec culture. In these calculations no allowance was made for the reduction in numbers due to the required decontamination procedures (likely to be about 2 logs), so that the true number was probably about $10^{10}$.
organisms. In cattle viable unit counts on solid media of about $10^6$ per gram (equivalent to about $10^8$ organisms per gram in faeces before processing) have been measured. Thus environmental contamination from even a single clinical case may be considerable. Moreover, *M. a. paratuberculosis* has been shown to survive for considerable periods in the environment (see above) so that levels of environmental contamination might build up over time.

Whilst clinically affected animals are likely to represent the greatest risk to herd mates, subclinically infected animals also shed *M. a. paratuberculosis* in faeces, and infection may persist in a herd or flock with few or no observed clinical cases. Experimental and field observations consistently show faecal shedding in clinically normal cattle. There has been less work in sheep due to the historical difficulties in culturing sheep strains but a recent prospective study in pastured sheep demonstrated excretion as early as 13 months of age, long before the appearance of clinical signs and before any serological response. There is also evidence from prospective studies that excretion can occur from animals that never become clinical cases or later eliminate the infection entirely. Faecal shedding of *M. a. paratuberculosis* was observed sporadically for the first 6 months after experimental infection in calves which showed no clinical signs out to 20 months. Another study demonstrated faecal shedding commencing 3-5 months after inoculation. Thereafter there was a steady decline in the proportion of cattle shedding the organism, except in those animals which were later shown to have extensive pathology. And in a field study, *M. a. paratuberculosis* was cultured from the faeces of 6 of 8 bulls 19 and 31 months after exposure to an infected pen-mate, after which time cultures were always negative. These animals had obviously become infected and excreted the organism, but did not progress to clinical disease. A recent study has shown that *M. a. paratuberculosis* can use normal host physiological mechanisms (via macrophages which digest apoptotic cells) to reach the lumen of the intestine without any damage to the host. This provides a mechanism for bacterial shedding at any stage of the disease. Therefore no infected animal should be considered a zero risk to cohorts.

There are also many experimental studies demonstrating passive excretion after oral infection. In a specific study the organism was isolated from faeces of heifers 18 hours to 7 days after orogastric intubation with the faeces from clinically affected cows (doses from $5 \times 10^6$ colony forming units (CFU)), peaking at days 3 and 4. The pattern of excretion was exactly the same as that seen for inert particulate matter. Passive excretion has also been noted incidentally during experimental inoculations in cattle and sheep, usually when large doses have
been administered. In natural situations positive faecal culture in the absence of other evidence of infection at necropsy suggests passive excretion. Such findings have been reported in deer, sheep and goats. There are some circumstances where passive excretion may have significant epidemiological consequences. Previously uninfected animals briefly exposed to high levels of environmental contamination (such as might occur in poorly disinfected saleyards or transport vehicles, or possibly on travelling stock routes) may excrete \textit{M. a. paratuberculosis} and present a risk to other stock for up to a week thereafter. Such animals are likely also to have been infected at the same time and may thus present a further risk when later they begin to excrete the organism themselves.

\textit{Infection via the milk:}
Several investigations have shown that subclinically infected cows can excrete \textit{M. a. paratuberculosis} in their milk. One study found that 22\% excreted the organism in their colostrum and 3\% in milk. Another found 12\% of subclinically infected cows shedding in milk and that prevalence of excretion in milk correlated with the level of faecal excretion. Clinically affected cows are even more likely to excrete the organism in milk, with one study showing a prevalence of 45\%. There are no references concerning the culture of \textit{M. a. paratuberculosis} from the milk of sheep. However, the situation is likely to be similar to that in cattle, and a recent study using PCR detected \textit{M. a. paratuberculosis} DNA in 88\% of milk samples from sheep with positive gamma-interferon (IFN-\(\gamma\)) tests. The numbers of organisms in infected milk may be low, although there are few references quantifying mammary excretion. A single study in subclinically infected cattle found only 2-8 CFU per 50 mL. Thus the significance of mammary excretion to the epidemiology of Johne's disease is unclear. That the highest levels of excretion are seen in colostrum is not surprising and is probably due to its containing large numbers of macrophages. Colostrum is available to the neonate at the time of highest susceptibility to infection (see below). Moreover, antibodies to \textit{M. a. paratuberculosis}, which may be present in colostrum of seropositive animals, have been shown experimentally to increase the uptake of \textit{M. a. paratuberculosis} by intestinal M cells. Labour-intensive management techniques are needed to avoid colostral intake and the use of pooled colostrum in dairy calves could potentially disseminate infection rapidly in a herd. Also pasteurisation does not completely remove \textit{M. a. paratuberculosis} from colostrum. In beef or sheep enterprises where neonates remain with their dams there may be significant concurrent oral exposure to faecally excreted organisms, so whether or not infection occurs via the milk has less practical implications.
**Congenital infection:**

Congenital infection is possible and infection of the foetus has been demonstrated frequently in cattle and at least once in sheep. Its epidemiological significance may be underrated. Most reports refer to foetal or uterine infection from clinically affected cows. The rate of foetal infection from such animals is surprisingly high, with figures of 26 to 35%, from subclinically infected cows rates of infection of foetuses of about 10% are found. Another study found no clinical or pathological evidence of Johne's disease in the dams of 20% of infected foetuses. There is only a single report confirming foetal infection in sheep, and a further report of antibodies to *M. a. paratuberculosis* in 3% of precolostral lambs from seropositive ewes. Whether foetal infection is rarer in sheep, or this is simply a reflection of less investigation is not clear. Also, ovine strains of *M. a. paratuberculosis* have until recently resisted most attempts at culture, which might lead to under-reporting of foetal infection in this species, given that foetal infection is usually without lesions. However, in all the above studies there is no indication whether any of the infected foetuses may have later become clinical cases, or even subclinical carriers. The ovine foetus has been reported to be unable to mount an immune response to certain antigens including BCG, and it is possible that this immaturity of the immune system extends also to *M. a. paratuberculosis*. As the immune system matures after birth it is possible that infection may be eliminated from some infected foetuses, or conversely that some degree of tolerance may occur, increasing the likelihood of development of a carrier state. This has not been studied.

**Venereal transmission:**

*M. a. paratuberculosis* has been isolated from the semen of clinically affected bulls and rams, and from the uterine fluids of clinically affected cows. Thus venereal transmission in either direction is theoretically possible, as is direct infection of a developing embryo, without established infection in the dam. However, such possible infections are probably of minimal significance in the field. Clearance of the organism from the uteri of cows after intra-uterine inoculation of high doses of *M. a. paratuberculosis* has been demonstrated, and infected bulls are likely to shed large numbers of organisms in faeces before significant numbers appear in the semen.

**Embryo transfer:**

*M. a. paratuberculosis* was isolated from uterine flush fluids from 3 of 4 clinically infected cows, and washed ova have been shown to harbour the organism indicating that embryo transfer from infected cows to uninfected donors is not entirely without risk (both to the developing embryo and the recipient cow).
Age and susceptibility to infection

Observations of natural and experimental infections in cattle over many years have indicated that calves are more susceptible to infection with *M. a. paratuberculosis* than are adult cattle. It is generally considered that most natural infection takes place in the neonatal period and that resistance to infection develops as animals age. While there is general agreement that adult cattle are resistant, the timing of development of resistance in calves is not clear. Hagan in 1938 reported increased susceptibility of neonatal calves when compared to older calves, and Taylor found six month old calves resistant compared to newborn or three month old animals. In contrast, Rankin found new born and six month old calves to be equally susceptible, while Larsen *et al.* found one month old calves to be more susceptible than 9 month old calves. Taken together these reports suggest that resistance in cattle probably develops between about 6 months and a year of age. This resistance shown by adult cattle is certainly not absolute, and moreover would appear to be resistance to the development of clinical disease rather than resistance to infection per se. This was shown in several experimental studies with large (180-200mg culture) oral doses of *M. a. paratuberculosis*. In one study one month old calves had more bacilli and lesions in their tissues 5 months after dosing than 9 month old calves or adult cattle, but most of the older cattle were nonetheless infected. Another study examined groups of 2 cows and 2 calves 2, 3, 4 and six months after dosing, and recovered *M. a. paratuberculosis* from 6 of the 8 calves, but from only one of 8 infected cows. However, cows examined at 2 months had more extensive lesions than the calves, while those examined later had fewer and less severe lesions, suggesting that an early host response in the cows was dealing with the infection, but nonetheless indicating that the cows had been infected. Studies of natural infections reinforce the experimental findings. Four of 6 adult cattle exposed naturally to a heavily contaminated environment had demonstrable infection in lymph nodes without detectable histological lesions, and 3 of 6 had excreted *M. a. paratuberculosis* in the faeces, but none developed clinical disease. Six of 8 bulls exposed at 16 to 27 months of age became infected, excreting the organism in faeces, but did not progress to clinical disease.

Less work has been published concerning age resistance in sheep, but the situation is likely to be analogous to that in cattle, with older sheep resistant to the development of clinical disease, but not necessarily to infection. Disease was produced in 8 of 9 experimentally infected lambs, 2 of 2 eight month old weaners, but in none of 8 adult ewes using an unquantified dose of intestinal material. Brotherston *et al.* found no difference in susceptibility as assessed by
culture and histopathology 2 to 12 months after experimental infection between sheep inoculated at 3 weeks or 3 months of age (total dose about $10^6$ organisms). Later studies by the same group reported no immunity to infection up to 20 months of age (total dose about $10^8$ organisms), but in this latter work examinations were carried out just 2 ½ months after first infection so there was no attempt to identify whether infected animals would later clear the infection or develop disease.

One aspect of epidemiological importance of the susceptibility of neonatal animals is that progression to clinical disease in adult animals is often associated with parturition or lactation. Thus increased excretion of infectious organisms occurs in close physical and temporal proximity to the arrival of new highly susceptible hosts.

Introduction and development of infection in a herd or flock

The most likely way infection is introduced into a herd or flock is with infected stock in the normal course of trade. Lateral spread from neighbouring properties via water or movement of contaminated soil is also possible, while straying stock or feral animals (such as goats which are poor respecters of fences) present an additional threat. Also the presence of M. a. paratuberculosis infection in various wildlife species provides another possible avenue for the spread or maintenance of infection in domestic stock. Active paratuberculosis has been demonstrated in many ruminant wildlife species, in rabbits and kangaroos (see above). In addition the possibility that other species may act as passive carriers must also be considered. Indeed, a number of Scottish wildlife species have recently been so implicated.

As discussed above there is little contention that within an infected flock clinical cases are the major source of environmental contamination for infection of other animals. But the subclinically infected animals cannot be ignored, particularly when planning strategies for the control of the disease. Clinical cases of paratuberculosis are just the “tip of the iceberg” with regard to infection in a herd or flock. Using evidence from sequential faecal cultures and necropsy follow-up in many dairy cattle herds over many years Whitlock examined the “iceberg” concept in detail. For each clinical case there may be 15-20 other infected animals, less than half of which might be detected by a single faecal culture. Some herds or flocks with low prevalence may rarely have clinical cases and/or animals may be culled from a herd before reaching the stage of clinical disease, so that infection may be completely unsuspected, and can only be detected by intensive sampling. For example 40% of 100 Dutch cattle herds free of clinical signs were confirmed infected when examined by a sensitive pooled
faecal culture technique repeated over four years. This paucity of clinical cases in herds with low levels of infection may be partly a function of the incubation period which may be many years. Incubation periods vary with the dose of infectious organisms and age at first exposure. Both experimental and field observations in cattle and sheep indicate that exposure to high numbers of *M. a. paratuberculosis* is associated with shorter incubation periods. When infection is first introduced into a flock environmental contamination and exposure of individual animals will be low. The disease progresses insidiously and it may be many years before clinical cases are seen. These may be confined to certain cohorts of animals, for example animals which were lambs or calves at the time of first exposure. Presumably the classical triad of host, pathogen and environment (which includes management strategies) has to come together for infection to establish and spread within a flock. Environmental factors favouring the exposure of young susceptible stock to large amounts of infected faeces are probably important, eg unsanitary conditions and high stocking densities. In less “favourable” environmental conditions Johne’s disease may never progress to become a significant problem in a herd, and infection may stabilise at very low prevalence.

**Strains of *M. a. paratuberculosis* – host specificity**

That several strains of *M. a. paratuberculosis* existed was suspected for many years because of the difficulty in isolating *M. a. paratuberculosis* from diseased sheep as compared to cattle, and the vivid yellow pigmentation of some isolates and lesions. Recently developed molecular biological techniques have confirmed these early suspicions. Studies using restriction fragment length polymorphism (RFLP) analysis on genomic DNA or IS900 PCR products have demonstrated that sheep are usually infected with S strains, and cattle with C strains. The S strains appear to be particularly host-adapted for sheep. They form a rather homogeneous group, although genetic differences among strains from different flocks of sheep in Morocco and South Africa have been described. The S strains have historically been difficult to isolate on routine media used for bovine isolates, and some S strains from the UK are strongly yellow pigmented. While mainly associated with sheep, natural infection with S strains occurs occasionally in goats, cattle and deer. Cattle have also been experimentally infected with presumed sheep strains. Bovine paratuberculosis in Iceland has been shown by PCR and restriction endonuclease analysis (REA) for IS1311 to be due to an S strain following introduction of the disease with infected sheep in 1933. Some culture negative paratuberculous Australian cattle were similarly shown to be infected with sheep strains. In each bovine case there had been contact of calves with paratuberculous sheep. Limited
epidemiological evidence suggests that transmission of S strains to cattle in Australia has been uncommon under extensive grazing conditions, whereas husbandry practices in Iceland appear to have favoured transmission of S strains to cattle. Also the Iceland experience suggests that the virulence of S strains in cattle may be considerably lower than in sheep.

The C strains are more varied and show less host adaptation. They are the common strains found in cattle and other species, including goats, and occasionally sheep. Sheep are also readily infected with the bovine strains in experimental situations (numerous authors). Australian isolates of *M. a. paratuberculosis* were examined using IS900 PCR and RFLP with a variety of enzymes and bovine isolates were commonly C1 or C3. All isolates from alpaca were type C1. Further work confirmed and extended these findings, and revealed variation among cattle isolates from different states in IS900 RFLP types. For example type C3 was the commonest in NSW cattle, but was completely absent from Victorian beef cattle. C1 was the most common in Victorian cattle, but not found in NSW cattle. The fact that such differences between states and types of enterprise occur is evidence that management factors can provide significant barriers to disease transmission, casting some doubt on the assumption that the observed differences in strains affecting sheep and cattle indicate differences in species susceptibility.

Another distinct strain (B strain) has been shown to infect Bison in Montana in the United States. Its epidemiology appears to be distinct from that in cattle and other farmed livestock. Evidence from field observations and experimental transmission indicates that the current Norwegian strain of *M. a. paratuberculosis* may also be a distinct strain. It causes disease in goats but has little or no pathogenicity for cattle. Moreover, when compared to isolates from Australia, New Zealand, Canada and the United States, two Norwegian goat strains did not hybridise to a *M. a. paratuberculosis* probe and had restriction patterns very different from those of other *M. a. paratuberculosis* strains.

*Pathology and clinical signs*

*Clinical findings*

The classical clinical sign of sheep with severe Johne's disease is progressive emaciation. Unlike cattle in which diarrhoea is common, only 20% of severely diseased sheep are so affected. These sheep usually have lesions in the large intestine. Because of the inability of the various specific tests to reliably identify infected animals, some attempts have been made to define
serum biochemical changes in infected animals. Clinically affected sheep have been shown to have low total serum protein and albumin levels, with normal gammaglobulin. Blood calcium is also low, and these serum biochemical changes are more marked in sheep with multibacillary disease. In cattle total serum protein (TPR), serum albumin, triglycerides (TRIG) and cholesterol were found to be reduced in experimental disease, while enzyme activities for creatine kinase (CK), fructose-1,6-diphosphate aldolase (ALD), lactate dehydrogenase, aspartate aminotransferase, $\alpha$-hydroxybutyrate dehydrogenase and alanine aminotransferase were elevated. TPR, TRIG, ALD and CK levels were considered to be of use in the diagnosis of Johne's disease, especially advanced cases in cattle. But no serum biochemical test has been particularly helpful in the diagnosis of subclinical disease in either species.

Gross lesions
Specific gross lesions are confined to the gastrointestinal tract but are not always recognised, even in sheep dying with Johne's disease (and having extensive microscopic pathology). Thickening of the intestinal wall, sometimes with corrugation of the mucosa is often present, usually most severe in the last few meters of the ileum and ileocaecal valve (ICV). Changes may extend forward as far as the duodenum in some sheep, or involve the proximal colon, and may be segmental with areas of normal intestine in between. The mucosal surface may have a granular appearance and is sometimes congested. In some countries, where pigmented strains of $M. a. paratuberculosis$ are involved, the thickened mucosa may have a yellow colour. On the serosal surface of affected intestines the lymphatic vessels are often dilated with a tortuous and corded appearance. Mesenteric nodes may be enlarged and usually are oedematous on the cut surface with little distinction between the cortex and medulla.

Histopathological lesions
Many authors have produced classification systems of varying complexity for the intestinal histopathology of Johne's disease in sheep, and these have been reviewed in detail by Clarke (1997). The most comprehensive system, which includes consideration of subclinical lesions, is that of Perez et al and this system will be specifically referred to in the discussion below.
In sheep with advanced clinical disease, two polar types of pathology have consistently been described.\textsuperscript{51, 65, 255, 261} Pathology in both types is extensive, involving intestine associated with and distant from the Peyer's patches (PP). The most common type is the multibacillary form (sometimes termed lepromatous, Perez type 3b), and this is also the most common presentation in goats.\textsuperscript{76} Pathology is dominated by diffuse infiltration of the lamina propria of the intestine with epithelioid macrophages containing large numbers of acid-fast bacilli (AFB). There is villous fusion and reduction in numbers of crypts, plus occasional crypt abscesses. Sometimes mucosal erosions are present and there may be varying infiltration with other inflammatory cells, mainly lymphocytes. Granulocyte infiltration is variable and often associated with small foci of necrosis. Giant cells are rare. It is not unusual for the epithelioid infiltration to extend to the submucosa. Oedema, lymphatic dilation and lymphocytic infiltration are often present in the submucosa and extend to the serosal lymphatics, which may also have granulomatous lesions. A lymphocytic neuritis has also been documented.\textsuperscript{259} Lesions in the draining mesenteric lymph nodes (MLN) consist of diffuse or multifocal epithelioid cell infiltration, mainly in the interfollicular areas of the cortex and paracortex and in the subcapsular sinuses. Acid-fast organisms are usually detectable, but in smaller numbers than in the intestine.

The other polar type of pathology in advanced disease is the paucibacillary form (sometimes termed tuberculoid, Perez type 3c). This would appear to be less common in severely affected sheep or in sheep dying of Johne's disease, with estimates of 12%,\textsuperscript{51} 15%,\textsuperscript{255} and 25%\textsuperscript{65} of reported cases. While the general changes of mucosal and submucosal inflammation and oedema are similar, cellular infiltration in these cases is dominated by lymphocytes, but there is always some accompanying multifocal infiltration by epithelioid macrophages. Giant cells are commonly present. Few AFB can be demonstrated in the intestinal lesions, and are absent in about 50% of cases. Lesions in MLN are similar to those of the multibacillary cases, but often contain focal granulomas containing giant cells or their degenerating remains, and AFB are rarely detected. Some authors describe necrosis and calcification associated with tuberculoid foci in naturally occurring\textsuperscript{261, 312} and experimental\textsuperscript{14, 241} Johne's disease, but this does not appear to be common, or can be ascribed to other causes such as parasitic infection.\textsuperscript{51, 65, 255} It appears to be associated with very high doses of organisms, often of bovine strains in the experimental situation.
This dual multibacillary/paucibacillary classification breaks down, however, when sheep with less severe or subclinical disease are considered, and of course some severely affected animals may also have intermediate lesions. Some subclinically affected sheep will have typical, multibacillary or paucibacillary, diffuse or locally extensive lesions as described above and can readily classified as such, but many have less extensive lesions as described below. In cattle the extreme polar forms are rarely reported, the number of AFB in lesions increases as the severity and extent of lesions increase, and giant cells are common in most lesions.

The mildest and least extensive of the described lesions of Johne's disease are those equivalent to the type1 and 2 lesions of Perez et al. There are no gross changes associated with such lesions. Type 1 lesions in naturally infected sheep are small focal epithelioid cell granulomas exclusively associated with intestinal, particularly ileal, PP. AFB are not detectable. Type 2 lesions appear to be an extension of the type 1 lesion. They are similar in character (multifocal small granulomas), but more widespread and involve also the intestinal mucosa overlying the PP. The mucosal lesions may be deep in the lamina propria and/or high in the villi. Sometimes small numbers of AFB are present in the mucosal lesions, but are rarely found in PP. In both type 1 and type 2 cases similar lesions may also be found in the MLN, but these are usually smaller, fewer in number and rarely contain detectable AFB. The type 1 and 2 lesions are often considered to be early lesions, in the light of similar pathology in experimentally infected sheep and calves. They appear in the first few months after experimental infection, but are often present long after the initial infection, so may also represent arrested lesions. Because these type 1 and type 2 lesions rarely contain AFB, they are often termed paucibacillary (or tuberculoid) cases. If the terms are interpreted in their broader sense as a description of interplay between infection and host immunity (and not simply as descriptions of numbers of organisms present) this terminology may not be appropriate and is possibly misleading especially when applied to early lesions.

The next step up in severity are the Perez type 3a lesions. They consist of multifocal epithelioid cell granulomas within the intestinal mucosa. These granulomas are larger, more widespread, both spatially associated with and distant from the PP, but not sufficiently severe to cause grossly visible changes. They are logically considered to be a further extension of the type 2 lesion and observations in experimentally infected sheep support this contention. A degree of lymphocytic involvement is described around blood vessels and lymphatics in the submucosa and serosa, and most cases have concurrent lesions in the MLN. The lymphangitis
may even be grossly apparent. AFB are detectable in most cases. Depending on the severity of the lesions, many such cases merge into milder or less extensive examples of the multibacillary or paucibacillary cases described above.

Extra-intestinal lesions of Johnne's disease are sometimes reported, but appear to have little pathogenetic significance and are more often ignored. Lesions in tonsil and retropharangeal nodes are sometimes reported, but rarely sought, and may be considered analogous to those in intestinal PP and MLN respectively. In more than half of severely affected sheep the liver has widespread small foci of epithelioid cells, occasionally with AFB, in the parenchyma. Lymphoid infiltration of the portal tracts may be present also. In 3% of such sheep similar granulomatous lesions were detected in peribronchial areas of the lungs, but there were no lesions in peripheral lymph nodes, spleen, kidney, heart, mammary glands, uterus or foetus.

Diagnosis of *M. avium* subsp. paratuberculosis infection

In the discussion of diagnostic tests for paratuberculosis sensitivity (the percentage of infected animals correctly identified as infected by the given test) and specificity (the percentage of uninfected animals correctly identified as uninfected) are often reported. It must be stressed that these should be considered against the background of the particular population of animals examined. Comparison of figures between different studies can be highly misleading unless factors such as the prevalence and stage of the disease within the population examined and the methods used to provide the “gold standard” against which tests are measured are considered.

Diagnostics tests can broadly be grouped into three main areas.

1. Detection of *M. a. paratuberculosis*. This includes cultural techniques and direct detection of organisms by staining, immunological and molecular methods.

2. Detection of host immune responses to infection, humoral or cell mediated.

3. The clinical and pathological features (discussed above), including biopsy in the live animal.

*Detection of Mycobacterium avium* subsp. *paratuberculosis*

*Culture:*

For the purposes of diagnosis culture is attempted mainly from faeces or from tissues obtained at necropsy (or rarely from biopsy). There is considerable variety of isolation methods and these were reviewed in detail in 1991. “Traditional” culture of *M. a. paratuberculosis* from faeces relies on initial decontamination using combinations of sedimentation, centrifugation, filtration and chemical disinfection with or without antibiotic treatment and incubation steps,
followed by culture on solid agar slopes containing mycobactin. Colonial morphology, slow growth and mycobactin dependency are used to identify isolates. Culture from tissues is similar, but depending on the particular tissue and method of collection may not require as stringent decontamination. Many American and Australian laboratories currently use decontamination methods based on Whitlock’s “double incubation centrifugation” technique. Briefly, the faecal sample is mixed with sterile saline and allowed to stand for 30 min. An aliquot of the surface fluid is transferred to 0.9% hexadecylpyridinium chloride (HPC) in half-strength brain heart infusion broth, incubated at 37 °C for 24 h, then centrifuged. The pellet is resuspended in solution containing antibiotics and incubated for 48 to 72 h at 37 °C. Aliquots are removed for inoculation onto solid media based on Herrold egg yolk medium (HEYM). In Europe many laboratories use disinfection in NAOH, oxalic acid and malachite green followed by culture on solid media based on Löwenstien Jensen (LJ) agar. The different methods for sample decontamination as currently used in American laboratories were summarised and compared by Stabel 1997, but there are no studies comparing the European and American techniques. These traditional culture techniques are adequate for culture of bovine strains of M. a. paratuberculosis but not for most sheep strains. There is one report of successful culture of ovine strains (which did not grow on traditional media) using solid media containing 50% egg yolk, and another indicating successful growth on LJ media without pyruvate. But only recently have ovine strains been reliably grown in vitro using modified Bactec 12B medium. This is a liquid medium containing 20% egg yolk, and growth is detected radiometrically. The identity of organisms yielding growth in radiometric media is confirmed by subculture onto solid media or by molecular methods (see below). Radiometric culture is also used for bovine samples and significantly reduces the time for detection of growth over that taken on solid media. Some authors report radiometric culture after filter concentration or Whitlock decontamination to be more sensitive than conventional culture for bovine faeces, whereas others have found no advantage. But all techniques are a balancing act between too little decontamination leading to contaminated cultures and too rigorous decontamination leading to false negative cultures. Reported contamination rates for faecal cultures range from about 4 to 11%. And, regardless of method, the decontamination of samples for culture will result in some loss of organisms. Several authors have measured or estimated this loss to be about 2 logs, using different culture techniques. This suggests that the limit of detection is about 100 organisms per gram of faeces. Thus a negative result on faecal culture indicates only that there were less than the detectable number of organisms in the faecal sample examined, not that the animal was not excreting the
organism. Detection of shedding by subclinically affected animals is often intermittent in both cattle and sheep. This is likely due to the small (and probably intermittent) numbers of organisms shed early in the disease process, uneven distribution of the organism in faecal samples, and the limit of detection of the culture techniques. Despite these reservations faecal culture remains the most sensitive routine test for detection of subclinical M. a. paratuberculosis infection in both cattle and sheep, returning positive results before the development of any antibody response. Pooled faecal culture is a more sensitive and cost-effective alternative to serological testing in sheep. Culture of M. a. paratuberculosis from tissues taken at necropsy is usually considered the most sensitive measure of M. a. paratuberculosis infection in cattle, but of course is not useful for routine in-herd diagnosis. In several studies histopathology at necropsy detected only 54% to 86% of culture-positive cattle or goats. In sheep, because of the historical difficulties associated with culture, intestinal histopathology has often been used as the gold standard. Recent experience indicates that culture of tissues is also more sensitive in sheep, with only 83% of culture-positive sheep having detectable lesions.

When considering the specificity of faecal culture, the occurrence of passive excretion of M. a. paratuberculosis is significant. Occasional animals in an affected herd or flock may be positive on faecal culture due to passive excretion, and have no evidence for infection at necropsy. Thus the specificity of faecal culture for M. a. paratuberculosis for an individual animal is less than 100%. However, if taken as a measure of infection in the herd, flock or property, a 100% specificity would be reasonable, assuming no problems with sample identification and accurate identification of any isolates.

Molecular techniques:
Considerable effort has gone into the development of sensitive alternatives to culture for the detection of M. a. paratuberculosis in the faeces of animals, to avoid the time delay inherent in all culture techniques. While some radiometric cultures may yield positive results in a few weeks, it can take many months to be sure of a negative result. The most widespread molecular technique for M. a. paratuberculosis diagnosis to date is PCR for IS900. IS900 was initially considered to be specific for M. a. paratuberculosis. However, recent work has revealed sequences very similar to IS900 in a number of as yet uncharacterised mycobacterium species. Thus REA analysis of the IS900 PCR product is necessary to avoid false positive results. In general PCR tests have been found to be less sensitive than faecal culture for bovine samples, but offer great advantages in terms of the speed of obtaining a result. While culture may detect faeces
containing 100 organisms per gram, the usual IS900 PCR requires about $10^4$ organisms per gram for a positive result from bovine faeces. Detection of about $10^3$ per gram has been claimed using a different, nested PCR for IS900. Recent work claimed sensitivities of 64 to 74% with direct-PCR and 44% with HC-PCR when compared to radiometric culture from sheep faeces. PCR is also used routinely for the rapid identification of isolates obtained from both conventional and radiometric cultures.

PCR appears to be more sensitive and reliable when applied to tissue samples. IS900 PCR for detection of *M. a. paratuberculosis* in tissues achieved similar sensitivity to culture for ileal tissues from heavily infected sheep. The test was less successful in detecting infection in lymph nodes, presumably because of the dilution by large amounts of host DNA. Modification of the IS900 PCR (alternate primers amplifying a shorter segment of DNA) for use in formalin-fixed paraffin-embedded sections has been described, but the test offered little advantage in routine diagnosis over simple Ziehl-Neelsen (ZN) stains, rarely returning a positive result in paucibacillary cases. But it does allow examination of archived samples and the specific confirmation of *M. a. paratuberculosis* infection when culture results are not available. IS900 PCR has also been used as a research tool on blood samples from infected animals, but has not found routine use to augment antemortem diagnosis. From heavily infected sheep PCR on blood had a similar sensitivity to the simpler and cheaper agar gel immunodiffusion test (AGID), detecting 66% of histologically confirmed multibacillary cases. In cattle, *M. a. paratuberculosis* DNA was detected by PCR in the blood and faeces of four of five clinically affected cows. When used in a herd of 72 infected cattle PCR on blood was positive in 60%, whereas only 30% were positive on a single faecal culture.

Molecular techniques have also been useful in distinguishing between different stains of *M. a. paratuberculosis*. Restriction fragment length polymorphism (RFLP) analysis on genomic DNA or IS900 PCR product has been used to differentiate sheep and cattle strains, and in some areas may be useful in the epidemiological study of outbreaks of Johne's disease. PCR for the IS1311 insertion sequence (present in both *M. avium* and *M. a. paratuberculosis*) followed by REA provides a simpler and cheaper alternative to RFLP analysis for the differentiation of sheep and cattle strains in Australia. A modified technique (amplifying a shorter segment) which works for formalin-fixed tissue allows analysis of archived samples.
In situ hybridisation with an IS900 M. a. paratuberculosis-specific probe has been used of for the detection of cell wall deficient forms of the organism in tissue sections. Acid fast forms were not labelled with this technique. It may prove useful in evaluating the significance of cell wall deficient forms of M. a. paratuberculosis in the pathogenesis of Johne's disease.

Stained smears:
Ziehl-Neelsen staining of faeces enables simple, quick and cheap diagnosis of Johne's disease. However it has very low sensitivity. Even in clinically affected animals only 30-66% may be detected, while in subclinically affected animals sensitivities of less than 20% are quoted. There are also doubts regarding specificity, since other AFB are not unusual in ruminant faeces, while Nocardia, Corynebacterium and Rhodococcus may also exhibit acid fast staining. ZN staining of histopathological sections has been discussed under pathology (above).

Immunoperoxidase labelling of histological sections:
This technique has been described for use mainly in experimental situations and has the theoretical advantage of revealing M. a. paratuberculosis antigens from cells which have lost their cell wall integrity and may not stain by the ZN method, providing advantages in the study of pathogenesis of Johne's disease. Mycobacterial antigens were observed by immunoperoxidase (IPX) staining in M cells of lambs soon after infection, but AFB were not seen in ZN stained sections. In another study the cytoplasm of cells (interpreted as follicular dendritic cells or macrophages) in the centres of intestinal PP follicles of infected bovine tissue was stained using IPX, although no AFB were detected in the same cells. IPX is considered by most authors to be more sensitive than ZN staining, especially when small numbers of organisms are present. When used with appropriate primary antibodies the technique is also more specific than ZN staining, allowing differentiation from M. bovis. Other reports indicate false positive results when using commercial polyclonal antibodies.

Detection of host immune responses
Humoral immune responses:
Many techniques have been used for the serodiagnosis of Johne's disease. These tests include the complement fixation test, the indirect fluorescent-antibody test, an indirect immunoperoxidase test, haemagglutination tests, enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) tests.

Currently ELISA tests are the standard serological tests used in cattle (mainly absorbed ELISA with M. phlei) and several commercial or in-house tests are in routine use in laboratories around the world. An enormous amount of work has been done and many dozens of papers have
been written on the optimisation of serological tests for bovine paratuberculosis. Specificity of commercial ELISA tests are consistently high when used in well characterised paratuberculosis-free cattle, with reported values ranging from 98.9 to 99.8%.\textsuperscript{222, 270, 363} In dairy cattle ELISA has also been used to detect antibodies in milk.\textsuperscript{240, 323}

In sheep the AGID is currently in routine use worldwide, with reported specificity of 99 to 100%.\textsuperscript{66, 93, 140, 219, 256, 292} ELISA tests have also been developed for use in sheep but despite advantages in ease of screening for large numbers of samples, have not replaced the AGID for routine diagnosis.\textsuperscript{92, 219} ELISA and AGID are reported to detect different subpopulations of infected sheep with only moderate overlap, so that the use of both tests in parallel will detect more infected animals.\textsuperscript{140} In an ELISA system sensitivity and specificity vary (in opposite directions) as the cut-off value is changed. When a cut-off to give specificity approaching 100% is used, sensitivity is no better than the AGID. Higher sensitivities can be obtained at the expense of specificity. This may be useful for identification of individual infected sheep in a highly infected flock, or to target sheep for further investigation in flocks of presumed low prevalence.

The differences between different serological tests pall into insignificance against the background of the pathogenesis of the disease. Simply, there is minimal humoral response until the disease is well advanced, so the sensitivity of tests for humoral antibody will always be limited unless the tests are applied to clinically diseased animals.\textsuperscript{222, 324} There is a consistent relationship between large numbers of AFB in lesions or heavy faecal shedding and positive findings in serological tests in sheep,\textsuperscript{66, 256} goats\textsuperscript{318} and cattle.\textsuperscript{87, 267, 324, 363} 95-100% of sheep with the multibacillary form of the disease were positive for antibodies to \textit{M. a. paratuberculosis} in AGID or ELISA tests whereas only 10-30% of sheep with paucibacillary lesions had positive serological results.\textsuperscript{66, 256} Sheep with only focal lesions were also rarely positive serologically. Prospective field studies and experimental infections consistently demonstrate faecal excretion before development of antibody response.\textsuperscript{53, 74, 209, 221}

Because the specificity of serological tests for \textit{M. a. paratuberculosis} infection is high, specificity is rarely of concern when the tests are used to identify infected animals within known infected herds. It becomes an issue, however when serological testing is used to screen herds of unknown infection status. Considerable angst and follow-up work ensue when a false positive serological result is obtained. Since many hundreds of animals from single flocks or herds may
be screened occasional false positives are unavoidable. The very close relationship between the ubiquitous *M. avium* and *M. a. paratuberculosis* has long been recognised as a potential problem for specificity, and the ongoing search for subspecies specific antigens to further increase the specificity of serological testing was recently reviewed by Stevenson *et al.*

The two organisms were shown to have negligible differences when used as antigens in an ELISA for bovine paratuberculosis. Recently a *M. a. paratuberculosis* specific protein (HspX) has been shown to be present in infected macrophages and offers some promise for more specific serological tests. However, it was recognised by sera from only 17% of infected cattle.

Other proteins uniquely expressed by *M. a. paratuberculosis*, the alkyl hydroperoxide reductases C and D, have been used in experimental ELISA to distinguish between *M. a. paratuberculosis* infected cattle and those infected with other mycobacteria, although again sensitivity was very poor.

**Cell Mediated Immune responses:**

It is now well recognised that immunological resistance and the earliest specific immunological responses to *M. a. paratuberculosis* infection depend on cell mediated immune mechanisms. Tests for cell-mediated immunity (CMI) include the intradermal skin test for delayed type hypersensitivity (DTH), lymphocyte stimulation assays, migration inhibition test, IFN-γ and interleukin(IL)-2R assay. Of these only skin testing and the IFN-γ assay have been much used outside of experimental situations. IL-2R assay is a recently developed test that shows promise for the detection of early infection, but so far has been used only in small numbers of experimentally infected goats. The intravenous Johnin test has been used in the past, either alone or to prime a subsequent lymphocyte stimulation assay.

In cattle skin testing is now rarely used because it has been found repeatedly to have both low sensitivity and low specificity. However, while most reports suggest that the sensitivity of the skin test is quite low in adult sheep, the specificity is reported to be high, both in the experimental situation and in the field. Little attention has been paid to lambs, but experimental work suggests that skin testing can be quite sensitive soon after infection, with levels declining over time.

The IFN-γ assay, which measures production of IFN-γ by leukocytes stimulated *in vitro*, has received considerable attention recently. It has been shown in a number of studies to be superior to tests for humoral antibody in the detection of subclinical infection in both sheep and cattle. Sensitivity estimates for the detection of subclinical paratuberculosis in cattle range from 50 to 93% (compared to repeated faecal culture results). Clinically affected
cattle may give negative results.\textsuperscript{302-311} The IFN-\(\gamma\) assay also shows promise for detection of infected animals soon after exposure. In several studies where lambs and kids were experimentally infected orally with large doses of \textit{M. a. paratuberculosis}, IFN-\(\gamma\) assays returned positive results after 9-18 weeks,\textsuperscript{130,314,318} and in goats the test reliably distinguished infected from uninfected animals during the first year after experimental infection.\textsuperscript{318}

Of particular interest in sheep is the relationship between pathological findings and CMI responses. Sheep with the diffuse paucibacillary lesions are commonly positive in IFN-\(\gamma\) and DTH tests (77 and 100\% respectively of such sheep were positive), whereas sheep with diffuse multibacillary lesions were less likely to be positive (36\% and 38\% of such sheep)\textsuperscript{257}. In addition most (88\% and 80\% respectively) sheep with lesions confined to the intestinal PP (which usually represent subclinically affected sheep) were positive in these tests.

In general specificity of tests for CMI is lower than that of tests for humoral antibody. Reported specificities for the IFN-\(\gamma\) assay are 97.6\% in cattle,\textsuperscript{39} and 93\%\textsuperscript{257} in sheep. Skin testing has very low specificity in cattle\textsuperscript{62} although in sheep some studies report 100\% specificity.\textsuperscript{257} Tests for CMI often use undefined antigen preparations such as purified protein derivatives which are likely to contain cross-reactive antigens which may be shared with other mycobacteria, \textit{Actinomyces} spp, \textit{Corynebacterium} spp, \textit{Nocardia} spp, \textit{Streptomyces} spp or fungi\textsuperscript{62}. Such organisms may also be more likely to stimulate CMI rather than humoral responses. Purified protein derivatives (PPD) prepared from either \textit{M. a. paratuberculosis} or \textit{M. avium} produced similar DTH reactions in sheep experimentally infected with either organism,\textsuperscript{156} and were without significant difference in the stimulation of lymphocytes for assay of IL-2R expression in infected goats.\textsuperscript{360} However, in experimental situations IFN-\(\gamma\) and lymphocyte stimulation assays using defined antigens specific to \textit{M. a. paratuberculosis} or \textit{Mycobacterium avium} subsp. \textit{sylvaticum} could differentiate sheep infected with either organism.\textsuperscript{49}

\textit{Pathology – biopsies from live animals}

The clinical and pathological findings in paratuberculosis have already been discussed. The use of biopsy samples for diagnosis in living animals has been described several times in cattle. In clinically affected cattle histopathological examination of MLN biopsies was considered to be as successful as more widespread histopathological examination after necropsy in the confirmation of paratuberculosis.\textsuperscript{254} The technique is not only useful in advanced disease, but shows promise for the diagnosis of subclinical and early infection. MLN biopsies examined histologically (detected 5/29) and by culture (detected 26/29) diagnosed infection in 29/223
cattle on a heavily infected farm, in which other diagnostic methods (serum complement fixation test, intradermal johnin test and microscopic examination of the faeces) had produced negative results. In another study of 12 subclinically affected cattle, biopsy of terminal ileum (TI) and MLN detected 92% by culture and 75% by histopathology. In experimentally infected calves 14 months after dosing, and before any immunological evidence of infection ileal and MLN biopsies detected 6/10 (4/10 biopsies were culture positive and 4/10 had histopathologic lesions). There are no reports of the use of biopsies to diagnose ovine paratuberculosis in the field, but experimental findings using both culture and histopathology after infection with a bovine strain suggest that this may be a useful technique.

Control

Vaccination

Field experience has consistently shown that vaccination reduces the prevalence of clinical disease in flocks of sheep and in cattle herds, but has not been able to eliminate the disease. Similarly vaccination of deer led to a reduction of clinical disease, but histopathological examination suggested that infection was still occurring. In contrast, vaccination of goats in Norway appeared to be more effective at eliminating infection. The national goat flock (131,000 animals over 15 years) was vaccinated with a live-attenuated vaccine, and the incidence of infection (based on post mortem examination of over 15,000 goats) was reduced from 53 to 1%. Moreover, the observed cases were almost exclusively in animals that missed vaccination or were too old at the time. Whether this effect is due to the diligence and scale of the program or a species or strain difference is not clear.

Experimental studies support these field observations. While vaccinated animals have lower levels of infection and less severe pathological changes, vaccination fails to eliminate or prevent infection. The protection afforded by vaccination is thus relative rather than absolute. Several studies have been published using sheep infected with bovine strains of M. a. paratuberculosis. In the 1960’s subcutaneous (SC) use of an adjuvanted killed vaccine in lambs reduced, but did not eliminate, viable counts in the intestinal mucosa from one to 9 months after infection, and did not prevent spread of the organism to the local lymph nodes. The incidence of lesions in vaccinated sheep was reduced and the lesions were fewer, more focal and tuberculoid in character than the more diffuse epitheliod infiltrates in unvaccinated sheep. Another study using live attenuated vaccines found similar pathological changes seven months post infection, with AFB and lesions in intestinal PP and MLN of vaccinated lambs, and again the intestinal lesions were reduced in number and size compared to those of unvaccinated lambs.
Similar results were also obtained in a recent study on lambs experimentally infected with ovine strains of *M. a. paratuberculosis*. Pathology in the vaccinated lambs was less severe than in non-vaccinated lambs and no AFB were seen in the vaccinated group.

There is evidence that protection afforded by vaccines is mediated by CMI. Lesions in vaccinated animals are similar to paucibacillary or focal lesions that have been associated with strong CMI responses in unvaccinated animals. Specific studies have shown correlation of the strength of DTH reactions after vaccination with the reduction in the level of infection in tissues at later necropsy. And many studies have documented the strong CMI responses that occur from about two months post-vaccination. Vaccination usually also produces strong humoral immune responses within a month of vaccination, but these responses are unlikely to be associated with protection.

There are several hypotheses for the poor performance of vaccination in controlling *M. a. paratuberculosis* infection. The usual SC route of administration, while inducing significant systemic CMI responses may be less effective in producing local CMI responses in the gut. And in an infected herd or flock, infection may often occur prior to vaccination. In an experiment designed to test these hypotheses systemic (peripheral blood mononuclear cells (PBMC) and spleen cells) and local (caecal lymph node) CMI responses (IFN-γ production by cells stimulated with *M. a. paratuberculosis* antigen) were measured at about 6 weeks of age in experimentally infected calves vaccinated before or after infection. Infected unvaccinated calves had elevated local but not systemic CMI responses. Vaccinated calves had strong systemic immune responses regardless of the timing of infection, and the local response was also significantly enhanced in calves vaccinated prior to infection. In calves vaccinated after infection the effect on local CMI was blunted, suggesting that prior infection had indeed reduced the effect of vaccination. Several studies have examined the effects of booster vaccination on older already infected animals. In both cattle and sheep revaccination did not enhance protection and sometimes led to clinical disease through the development of a massive cellular response.

Other management options

Establishing and maintaining herds free of infection:

Elimination of Johne's disease by destocking, allowing time for pastures to lose their infectivity, followed by restocking with uninfected animals is theoretically possible, and has been attempted in some Australian sheep enterprises. Potential problems with such an approach are
the difficulty in ascertaining the true status of replacement animals, possible reinfection from neighbouring infected properties, the unknown time before pastures are truly “safe”, and of course the financial implications for affected farmers. The costs of such programs are considered too great in most areas where Johne's disease is endemic and widespread.62

Various certification or market assurance programs are used in many countries to identify herds and flocks as free (or at least of reduced prevalence) of Johne's disease.62 372 These use a number of strategies from whole herd testing to targeted sampling, with pooled or individual faecal cultures and/or serology, repeated at defined intervals, usually from 6 months to two years. There may also be requirements for appropriate fencing, control of straying stock or feral animals and approved sources of uninfected replacement animals.

Culling of infected animals:
This control option is limited by the accuracy of the available diagnostic tests to detect subclinical infection. Removal of clinical cases in cattle herds is a logical first step in reducing environmental contamination, but as discussed above is simply dealing with the tip of the iceberg. Culling on the basis of serological results is only marginally better. Culling on the basis of tests for CMI may be more effective since tests for CMI detect more animals early in the disease process than serological tests. Use of tests for both humoral and cell mediated immunity used in combination will detect more infected animals.257 The IFN-γ assay may be useful on an individual animal basis to remove infected animals from cattle herds.311 One note of caution should be sounded on the use of CMI tests in test and cull programs. Studies have revealed that sheep with the highest levels of CMI are later found to have the lowest levels of infection.112 131 Culling on CMI results might inadvertently remove the more resistant animals from the population – an undesirable outcome if the resistance is partly genetically based. Culling faecal culture positive animals does not have these concerns and has been shown to significantly reduce the number of cattle shedding at subsequent tests.153 Remaining animals were mainly low shedders, and moreover infected heifers were not detected in follow-up tests, indicating reduced exposure and a prolongation of incubation period. Because of the risk of vertical transmission the offspring of known infected animals should also be culled.62

Management of young stock:
Detailed protocols for the management of calves to minimise infection from adult stock involve separation at birth, feeding pasteurised milk replacer, separation from adults until of productive age and general hygiene considerations.62 Combined with a test and cull program the prevalence of disease in a herd may be greatly reduced.
Selection of resistant animals:

Selection for genetically resistant animals has not been investigated in any detail. No consistent breed associations have been found in either sheep or cattle. An extensive study of the pedigree records for over 7000 cattle found only a small genetic influence on the susceptibility of cattle to infection with *M. a. paratuberculosis* and concluded that traditional selection for resistance would be inefficient.162

Treatment of clinically affected animals

Treatment using similar drug combinations to those used for the treatment of mycobacterial disease in humans is theoretically possible but rarely considered in animals, because of the expense and time involved. Treatment of experimentally infected calves using a combination of rifampicin, streptomycin and pyrazinamide given orally daily for seven months was apparently successful.32 Similar therapy has also been used in an infected rhinoceros in an Australian Zoo,(D Blyde, personal communication) and antimycobacterial drugs were effective in treating four clinically affected stumptail macaques.208

Pathogenesis

Overview

The broad events in the pathogenesis of Johne’s disease have been well recognised for many years, but the detail remains an area of considerable confusion despite a number of recent studies. Usually infection takes place after ingestion of contaminated material by young susceptible animals. *M. a. paratuberculosis* organisms then enter the host via the lymphoepithelial tissues of the intestinal PP and are rapidly taken up by macrophages, which localise in the PP, the overlying intestinal mucosa and regional MLN. There they may resist the innate defences of the macrophage and multiply intracellularly, stimulating a primarily cell-mediated Th1 type immune response. Macrophage activity is upregulated by cytokines (especially IFN-γ) released from activated T lymphocytes, but *M. a. paratuberculosis* may persist, and microscopic focal lesions may develop. *M. a. paratuberculosis* may continue to multiply, and after a variable period (usually years) the host’s CMI responses are unable to contain the infection. Lesions increase in extent and may become grossly visible. Their microscopic appearance and clinical significance is dependent on host immune reactions. Animals with paucibacillary lesions have well developed Th 1 immune responses, but although uncontrolled multiplication of *M. a. paratuberculosis* is prevented the organism survives and severe immunopathology may be responsible for clinical disease in some animals. In animals which develop multibacillary lesions a switch from a Th 1 to Th 2 type of immune response occurs, *M. a. paratuberculosis* multiplies in an uncontrolled state, and non-protective humoral antibody may appear. Clinical signs may be precipitated by the destruction of large areas of absorptive mucosa and by the release of pro-
inflammatory cytokines. The following table briefly summarises the usual course of these events and the usual criteria (pathology, culture from organs, CMI, antibody levels, and faecal excretion) which can be used for diagnosis.

<table>
<thead>
<tr>
<th>Stage of disease</th>
<th>Age</th>
<th>Time after infection</th>
<th>Pathology</th>
<th>Positive organ culture</th>
<th>Cell mediated immunit y</th>
<th>Humoral immunit y</th>
<th>Faecal excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>first infection</td>
<td>neonate</td>
<td>weeks</td>
<td>normal</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>subclinical infection “silent”</td>
<td>juvenile to adult</td>
<td>weeks to years</td>
<td>normal to focal (type 1 and 2)</td>
<td>variable</td>
<td>variable to high</td>
<td>none</td>
<td>none to low</td>
</tr>
<tr>
<td>subclinical infection “shedder”</td>
<td>juvenile to adult</td>
<td>months to years</td>
<td>focal to diffuse</td>
<td>high</td>
<td>variable to high</td>
<td>low</td>
<td>variable</td>
</tr>
<tr>
<td>Clinical disease</td>
<td>adult</td>
<td>years</td>
<td>extensive diffuse paucibacillary</td>
<td>high</td>
<td>high to very high</td>
<td>low</td>
<td>variable (excrete small numbers)</td>
</tr>
<tr>
<td>Clinical disease (usual form in severe cases)</td>
<td>adult</td>
<td>years</td>
<td>extensive diffuse multibacillary</td>
<td>very high</td>
<td>variable</td>
<td>high to very high</td>
<td>very high (excrete large numbers)</td>
</tr>
<tr>
<td>Recovered</td>
<td>juvenile to adult</td>
<td>weeks to years</td>
<td>normal to focal (type 1 and 2)</td>
<td>none</td>
<td>variable?</td>
<td>none</td>
<td>none – passive only</td>
</tr>
</tbody>
</table>

In the above table terms mean:
- none (occasional animal positive when tested on a single occasion, <5%)
- low (small numbers of animals test positive, 5 – 25%)
- variable (reported values vary, 25 - 75% test positive)
- high (many animals test positive, 75 – 95%)
- very high (almost all animals test positive, >95%)

The pathogenesis of other mycobacterial infections such as tuberculosis, leprosy and *Mycobacterium avium* complex infections in man has been extensively studied. Tuberculosis in particular has received a considerable resurgence of attention recently. It is estimated that one third of the world’s population is currently infected, while the emergence of multiple drug resistance and the increased susceptibility of people with concurrent HIV infection have the potential to markedly exacerbate the problem. A rapidly expanding area in the study of mycobacterial pathogenesis is the identification of mycobacterial genes specifically expressed during infection. Such genes most likely encode products required for survival within the host and for progressive infection. Particular progress is being made with *M. tuberculosis*, for which the complete gene sequence is now known. This information is expected to provide new strategies to prevent tuberculosis infection, new targets for antimicrobial therapy and new.
insights into the infectious process. This work may have important applications in the study of the pathogenesis of paratuberculosis in ruminants.

Entry to the host - route of infection

The usual route of infection in the natural disease is ingestion of organisms from faecally contaminated pasture/soil, from faecal contamination of the udder or via the milk. The relative importance of these natural routes of infection is discussed under epidemiology. Most recent experimental infections in ruminants have been done using the oral route and oral infection will be assumed in discussion in following sections unless otherwise specified. But other routes of infection are possible and their study offers some insights into pathogenesis. Congenital and venereal infection are discussed under epidemiology, but there have been no studies on the likely outcomes of such infections.

A number of investigations in ruminants have used parenteral infection. IV infection in sheep produced detectable lesions in the intestine earlier than oral inoculation and also produced more severe lesions, while calves infected IV had higher colony counts than those exposed either orally or SC. Regardless of the route of infection, after several months the highest levels of \textit{M. a. paratuberculosis} were found in the intestine and mesenteric nodes indicating the preferential localisation to these areas. Lesions followed a similar pattern. Additional lesions according to route of infection, eg prescapular lymphadenopathy in SC inoculated calves, and pulmonary lesions following intratrachael inoculation in sheep were also seen, but many of these were likely to be the result of the sheer bulk of foreign material introduced, rather than infection \textit{per se}. Overall, these investigations suggest that in cases of natural infection by other than the oral route, similar progression of disease could be expected. Similar conclusions are drawn from studies using laboratory animal models, as discussed under host range.

Penetration of the mucosal barrier

Early experimental studies using mycobacterial culture and histopathology provided evidence for uptake of \textit{M. a. paratuberculosis} by the intestinal mucosa overlying the PP, particularly in the ileum, of both sheep and cattle. The organism was recovered from intestinal mucosa, but not MLN of sheep, 12 and 36 hours after oral dosing, and similar results were obtained in experiments with hamsters. Lesions are rarely seen in animals in the first few months after experimental infection, but when present support an association with mucosal lymphoid tissue of the intestine and particularly the ileum. They consist of accumulations of epithelioid cells closely associated with, and sometimes within, the follicles of the PP. Similar results were
obtained in calves using very large doses (200 mg culture) and in these experiments the tonsil was also shown to be an important site of entry. Lesions consisting of occasional giant cells and groups of epithelial cells in the tonsil, PP of ileum and associated nodes were present in some calves 2 months after infection. A pathogenesis similar to that of tuberculosis was postulated with a “primary complex” in the tonsil and retropharangeal nodes, or in the intestine and MLN. This study also demonstrated wide spread of \( M. a. paratuberculosis \) throughout the body in the first 2 weeks after infection, with positive culture results from peripheral lymph nodes, liver, spleen and kidney. In a complementary experiment radioactive particles of similar size to \( M. a. paratuberculosis \) were shown to enter the tonsil and PP and to be distributed throughout the reticuloendothelial system in a similar pattern. Whether this systemic spread is a reflection of the very high doses used or a normal occurrence early in natural infection is not clear. Infected macrophages are potentially quite mobile cells, as evidenced by the localisation of infection in the gastrointestinal tract after infection by novel routes, and the systemic spread of the organism that can occur in advanced disease. \( M. a. paratuberculosis \) has been demonstrated by culture or PCR in mononuclear cells from blood, tissue fluids and from other non-intestinal tissues including spleen, liver, kidney, udder and peripheral LN from clinically affected cows. However, lesions in tissues outside the gastrointestinal tract are rare and any systemic spread may be of minimal pathogenetic significance.

Recent studies have further localised the uptake of \( M. a. paratuberculosis \) to the lymphoepithelial tissue of the intestinal PP, and the pivotal role of the mucosa-associated lymphoid tissues as sites for uptake of pathogenic mycobacteria has been recently reviewed. Experiments using ligated ileal loops in calves demonstrated that both live and heat-killed \( M. a. paratuberculosis \) were present in both the M cells and in intra- and subepithelial macrophages 20 hours after infection. Macrophages containing mycobacteria were also observed deep in the follicles of the underlying PP. No degeneration of the M cells was seen, and they also displayed vacuoles containing electron-dense material interpreted as degraded bacilli. These results suggest that \( M. a. paratuberculosis \) crosses the intestinal epithelium by M cell uptake, rather than by active invasion. A further interesting observation was that the addition of anti-\( M. a. paratuberculosis \) serum to the inoculum increased the uptake of organisms. Thus it is possible that antibodies ingested in the milk from seropositive cows may actually facilitate uptake of the organism in suckling calves. Experiments in sheep using oral infection with \( 5 \times 10^{10} \) organisms of the ovine strain gave similar results. \( M. a. paratuberculosis \) antigens were demonstrated by IPX in the ileal PP and sporadically in the jejunal PP of all lambs from 12 hours to 14 days after infection,
peaking at 7 days. No organisms were detected by ZN staining. The antigens were located mainly in the epithelial cells associated with the domes of the PP, and occasionally in macrophages. The failure to visualise organisms with ZN stains suggests that many organisms are degraded in the M cells and macrophages or may exist as L forms. 106 In this study the numbers of organisms arriving at the ileal mucosa may have been considerably less than in the calf studies where 10^9 organisms were injected directly into isolated ileal loops. In both these studies there is evidence that many of the M. a. paratuberculosis ingested by macrophages are degraded. However, recent studies in goats, which confirm the role of M cells in the uptake of M. a. paratuberculosis, demonstrated intact AFB in M cells and in leukocytes in the M cell pocket, in the domes and the underlying submucosal interfollicular areas of the PP. 107 Bacilli in the domes were also frequently seen extracellularly, whereas phagocytes had engulfed most of the bacteria in the submucosal interfollicular areas. Examination just one hour post infection probably explains both the retention of acid fastness of many bacilli and the occurrence of free bacteria in the domes. In vitro studies complement the above work, providing ample evidence that ruminant macrophages have considerable ability to ingest M. a. paratuberculosis. 56 100 103 106

The tonsil has a similar lymphoepithelial relationship to that of the PP, 248 (p488) and should not be overlooked as an alternate portal of entry. To establish infection via the intestinal PP, orally acquired M. a. paratuberculosis must first survive the non-specific defences of the alimentary tract (microbial competition of commensal organisms, peristalsis, acidity of the abomasum, digestive enzymes, effect of bile, lactoferrin, mucus). 64 Organisms invading via the tonsil avoid these non-specific protective mechanisms, so the effective dose reaching the tonsil may be much higher than that lower in the tract. Tonsillar or retropharangeal lymph node infection has been demonstrated in a number of studies, 115 117 250 251 but is often not investigated and even totally avoided in experimental infections which utilise gastric intubation.

The ileal PP of ruminants is quite distinct from the other intestinal PP, and from the ileal PP of other mammals. It is the major site for B-cell development and production (antigen-independent mutation of Ig genes, 248 (p512)) reaching its greatest development soon after birth and involuting from about 6 months of age. Anatomically the ileal PP contains abundant lymphoid follicles (mainly B cells) with little parafollicular tissue (mainly T cells) and this is reflected in the relative proportions of T cells in the tissue overall (1-2% for ileal PP compared to 30-50% in the jejunal PP (Miyasaka et al, cited by Lugton. 197) Greatest levels of macromolecular antigen uptake in the intestine have been demonstrated for the follicle-
associated epithelium of the specialised ileal PP, although classical M cells are not present.\(^{248}\) (p 524) It is also the area where most severe lesions of Johne's disease are usually seen, particularly in sheep. Is this a coincidence or do the anatomical and immunological particularities of the ruminant ileal PP contribute to both the particular susceptibility of ruminants to paratuberculosis and the development of age-related resistance to infection?\(^{150}\)

In summary, uptake of *M. a. paratuberculosis* appears to be an active process of the mucosa associated lymphoid tissue. This uptake appears not to be dependent on any pathogenic properties of the organism since killed organisms and similar sized inert particles are similarly taken up.

*Survival in macrophages*

Having traversed the lymphoepithelial cells of the ileum or elsewhere, and been taken up into phagosomes of macrophages, establishment of infection requires that *M. a. paratuberculosis* evades the bactericidal mechanisms of these cells.

These mechanisms include destruction by enzymatic (eg lysozyme) degradation or acid production in mature phagosomes, or by production of reactive oxygen intermediates (eg superoxide, $\text{H}_2\text{O}_2$) and reactive nitrogen intermediates (eg nitric oxide (NO)).\(^5^7\) Growth can also be inhibited by the restriction of available metabolites, particularly iron, within the phagosomes of the macrophage.\(^1^2^1^\) \(^1^8^9^\) Macrophages are also important antigen presenting cells (APC), presenting mycobacterial antigens to T lymphocytes, stimulating specific adaptive immunity. Mycobacteria are more readily killed by macrophages activated by cytokines such as IFN-\(\gamma\), which is of particular significance as acquired immunity develops.\(^7^5^\) Thus another protective strategy is apoptosis (programmed cell death) of the macrophage or lysis by activated lymphocytes, releasing mycobacteria for phagocytosis by other more activated macrophages.

The mechanisms for the increased bactericidal ability of IFN-\(\gamma\) activated macrophages are many, including facilitated maturation of phagosomes to phagolysosomes,\(^7^5^\) reduction of iron levels in phagosomes,\(^1^2^1^\) and increased release of NO.\(^3^7^9^\) Maturation of macrophages also leads to morphological changes. Epithelioid and giant cells store less iron than less differentiated macrophages, and this may be one mechanism for reduced survival and multiplication of *M. a. paratuberculosis* in the paucibacillary forms of Johne's disease.\(^1^8^9^\)

Mycobacteria in general have evolved considerable abilities to evade these destructive mechanisms.\(^1^2^3^\) \(^2^6^8^\) Many studies have demonstrated the survival of *M. a. paratuberculosis* in
particular within ruminant phagocytic cells. *In vitro* studies using radiometric culture have demonstrated multiplication of *M. a. paratuberculosis* within bovine monocytes or monocyte-derived macrophages, and electron microscopic studies have shown that *M. a. paratuberculosis* remained intact within macrophages cultured from both clinically healthy and infected cows. Moreover, *M. a. paratuberculosis* survived in IFN-γ activated macrophages cultured from neonatal bovine bone marrow, whereas those same macrophages readily killed *Listeria monocytogenes*.

Survival is not an all or nothing process, however. Other *in vitro* studies have demonstrated that multiplication and death of *M. a. paratuberculosis* occur simultaneously in infected bovine monocytes, and this is consistent with the conclusions from *in vivo* infection studies which demonstrate degraded organisms in macrophages. Early studies using organ culture from experimentally infected sheep support the contention that many infecting organisms may initially be destroyed by early host defences. *M. a. paratuberculosis* was recovered from intestinal mucosa of sheep, 12 and 36 hours after oral infection but could not be isolated from sheep killed after 7 days. However, infections were again demonstrated in the intestinal mucosa and MLN after 28-56 days in most sheep. These results indicated that *M. a. paratuberculosis* was readily taken up by the intestinal mucosa, and although many were subsequently inhibited by the host, some survive and later multiply to produce progressive infection.

Mycobacteria show resistance to the effects of reactive oxygen and nitrogen metabolites. It is also apparent that mycobacteria are able to avoid the induction of toxic oxygen molecules in macrophages by their use of the complement receptor CR3 in the initial phagocytosis by the macrophage. Ligation of this receptor fails to initiate respiratory burst activity, unlike other surface receptors. *In vitro* studies have shown that even IFN-γ stimulated bovine macrophages do not produce sufficient NO to limit *M. a. paratuberculosis* multiplication. *M. a. paratuberculosis* organisms express high levels of the detoxifying enzymes alkyl hydroperoxide reductases C and D, which provide protection against these reactive metabolites, whereas such expression is not seen in *M. avium*. Also, recent studies in mice with *M. avium*-infected macrophages have shown that NO may even exacerbate mycobacterial infection by causing the suppression of the immune response.

A recent review of the survival of mycobacteria in phagosomes and in particular the role of mycobacterial cell wall lipids suggests that, rather than blocking phagosome-lysosome fusion *per
se, mycobacteria may arrest phagosome maturation at a developmental level that does not permit fusion with lysosomal compartments.\textsuperscript{268} Arrest of phagosome maturation may also explain some of the immunosuppressive effects of mycobacteria. Mycobacteria-containing phagosomes are sequestered from intracellular trafficking pathways of major histocompatibility (MHC) class II molecules thus interfering with mycobacterial antigen presentation. Recent studies suggest that the greater virulence of \textit{M. a. paratuberculosis} compared to \textit{M. avium} subsp. \textit{avium} is dependent on this capacity to suppress mycobacterial antigen presentation to T lymphocytes. \textit{In vitro} infection of bovine macrophages with \textit{M. a. paratuberculosis} caused downregulation of expression of both MHC class I and class II molecules on the macrophage surface to a much greater degree than \textit{M. avium} subsp. \textit{avium}. Moreover, \textit{M. a. paratuberculosis}-infected macrophages, unlike those infected with \textit{M. avium}, were unable to upregulate MHC expression after activation by IFN-\gamma, and were not lysed by primed autologous lymphocytes.\textsuperscript{355} The effect is probably due to a cell wall fraction, and can be caused by heat-killed as well as live organisms. This is consistent with previous studies which have shown that mycobacterial lipoarabinomannan (LAM) can inhibit the IFN-\gamma mediated activation of murine macrophages.\textsuperscript{293} Similar results were seen when \textit{M. a. paratuberculosis} infected epithelioid macrophages from clinically affected sheep were examined by IPX. Their surface expression of MHC class II antigens was much reduced compared to those from normal sheep, but interestingly there was little difference between infected macrophages from multibacillary or paucibacillary cases.\textsuperscript{10} A recent \textit{in vitro} study of \textit{M. tuberculosis}-infected mouse cells identified a specific mycobacterial 19-kDa lipoprotein acting via Toll-like receptor 2 on macrophages as the cause of decreased MHC II expression.\textsuperscript{242}

Many of the studies discussed above have used macrophages cultured \textit{in vitro}. While providing useful insights into particular aspects of pathogenesis, we cannot expect them to reveal the whole story. In an infected animal macrophages are simultaneously exposed to a wide range of influences, including cytokines, hormones, neural effects, immunoglobins, and immunoreactive cells. Such effects will vary with the general state of health, with age, with reproductive status, with the genetic constitution, with other coexisting infectious and/or inflammatory processes, and with the development of immune responsiveness to the invading organisms. Also, we need caution in extrapolating results from experimental rodents to other species, or from one mycobacterial species to another. For example, IFN-\gamma activated murine macrophages can destroy \textit{M. tuberculosis} completely, whereas the same is not true for human macrophages.\textsuperscript{275} and
M. avium is less susceptible to the bactericidal effects of activated murine macrophages than is M. tuberculosis.245

Host immunological responses

The response to initial infection with M. a. paratuberculosis is dependent on the size of the infective dose and the immunity of the host. A sufficiently small dose in an animal with adequate innate immunity might be overcome by the non-specific defences of the intestine and local macrophages, and infection may be aborted without ever having initiated a specific immune response. Naive CD4+ T cell responses do not occur unless a high density/dose of antigen is present to cross-link the T cell receptor (TCR)321 and moreover M. a. paratuberculosis has been shown to decrease MHC expression by macrophages.355 This earliest stage is analogous to the classical stage one of pulmonary tuberculosis in which resident alveolar macrophages attempt to destroy small numbers of tubercle bacilli during the first week after infection.84

If the number of organisms ingested is larger or if the host’s innate responses are impaired, then the local macrophages will be unable to destroy the phagocytosed M. a. paratuberculosis, and significant bacterial multiplication within macrophages occurs. (Equivalent to stage 2 of tuberculosis in which logarithmic growth occurs within macrophages which are not sufficiently activated to destroy the mycobacteria.84) Macrophages may be lysed (by the direct effects of bacteria or by cytotoxic cells) and release bacteria, or infected macrophages may themselves divide. Dividing macrophages containing AFB have been seen in cases of ovine paratuberculosis.261 Blood borne monocytes (immature macrophages) are attracted by cytokines released by infected cells or by bacterial products, and ingest any released bacteria. By this stage sufficient antigen may be present for the initiation of specific immune responses, and sufficient organisms may be present for detection of infection by culture of intestinal tissues. It is highly likely that even at this early stage migration of infected macrophages to the regional MLN occurs.26

The subsequent development of specific immunity might lead to complete elimination of the infection, might restrict infection at a subclinical level (with the possibility of later exacerbation), or be insufficient for protection and progressive disease will develop.

Innate immune responses:

These are of particular importance in the early stages of infection and include phagocytosis and attempted destruction of organisms by macrophages as already described. A number of host
responses are involved in enhancing these innate defence mechanisms of macrophages. Firstly, the local intestinal macrophages may have already been non-specifically activated by a variety of local or systemic stimuli making them more effective at mycobacterial killing. This is likely to be the case for the local macrophages of older animals. In a recent review Cooper et al discuss the numerous “danger” signals recognised by cells important in the innate immune response. These signals include unusual bacterial carbohydrates and bacterial DNA, and the cells involved include not only macrophages, but also dendritic cells, NK cells, NK-T cells, γδ T cells, neutrophils and marginal zone B cells.75

Only very recently have these earliest immune responses in the gut of M. a. paratuberculosis infected animals been the subject of detailed studies. When experimentally infected lambs were examined up to 8 weeks post-infection, there were no consistent changes in IFN-γ production or lymphocyte proliferation of intestinal lymphocytes when stimulated in vitro with mycobacterial antigens, suggesting that specific immune responses had not yet developed. But intestinal PP and MLN from infected animals had higher proportions of CD4+, CD8+, CD2+ and γδ T lymphocytes, and numbers of B cells as well as circulating IgG were decreased, changes consistent with the notion of a developing early cell mediated rather than humoral response.26 Changes in levels of messenger RNA (mRNA) for various cytokines were also observed, but were not so simply interpreted. Tumour necrosis factor (TNF)-α levels were increased in MLN, and granulocyte macrophage colony stimulating factor (GM-CSF) levels were increased whereas IL-4 and IFN-γ levels were decreased in intestinal PP. This same research team using in vitro infection of cultured ovine alveolar macrophages demonstrated induction of mRNAs for the pro-inflammatory cytokines IFN-γ, IL-1β, IL-4, IL-6 and TNF-α, but not for the Th1-associated cytokines GM-CSF, IL-2 or IL-12.25 TNF-α is secreted by murine macrophages in direct response to phagocytosis of M. avium and can act in an autocrine manner priming the macrophage for antibacterial activity. The same is likely to occur in paratuberculosis, and indeed increased levels were demonstrated in the above studies both in vivo and in vitro. Interestingly, M. a. paratuberculosis was recovered in culture from the MLN of only 3, and from the gut of only one, of the 8 infected lambs, despite their having been dosed with in excess of 10⁹ CFU, suggesting that some control of the infection was indeed occurring. Murine and human macrophages can also produce IL-12 in direct response to mycobacterial infection as can neutrophils exposed to mycobacterial LAM, and this in concert with TNF-α can induce NK cells to produce IFN-γ which in turn activates macrophages.52 75 Although IL-
12 production has not yet been demonstrated in early *M. a. paratuberculosis* infections, administration of exogenous IL-12 concurrently with vaccination was shown to augment the IFN-γ response in MLN of experimentally infected calves.58

CD1 is a system of antigen presenting molecules expressed on the surface of APC including macrophages, is independent of the MHC system and is non-polymorphic. There is evidence that CD1 molecules are involved in the presentation of lipid and glycolipid antigens to T cells leading to specific adaptive immune responses.280 Mycobacterial LAM can be presented to T cells by CD1 molecules on human dendritic cells in leprosy and the expression of CD1 correlated directly with effective immunity to *M. leprae*.294 295 In vitro studies suggest that infection with virulent *M. tuberculosis* causes down-regulation of CD1 expression on APC's, pointing to another immune evasion mechanism by mycobacteria.341 Changes in CD1 expression have not been investigated in the adaptive immune response of ruminants to paratuberculosis. The CD1 system is involved also in the innate immune response via antigen independent recognition and lysis of cells expressing CD1 by γδ T cells and NK-T cells, providing a rapid response in early phases of infection.341 In the only study to date of CD1 involvement in paratuberculosis no changes in CD1 expression were found in infected lambs one month after infection, although increased numbers of γδ T cells were demonstrated in PP.24

In comparison with mice and humans, ruminants and especially young ruminants have high numbers of γδ T cells in the circulation and the intestinal mucosa, suggesting a particular role for these cells in early immune protection at mucosal surfaces.248(p517) Several studies already discussed have demonstrated increased numbers of γδ T cells in intestinal PP or MLN of lambs in the first weeks post-infection.24 26 In vitro studies of IL-2R expression in PBMC from experimentally infected goats provide some further evidence of a role for γδ T cells in early infection. After 120 hours of stimulation the levels of IL-2R expression by γδ T cells from uninfected goats approached that from the infected goats, possibly indicating activation due to a first line of defence against mycobacterial antigens.317 However, γδ T cells have traditionally been considered to have a suppressor function.142 Studies using γδ-knockout mice failed to demonstrate a protective role for these cells in *M. a. paratuberculosis* infections but did suggest a crucial role in the development of epithelioid granulomas similar to those seen consistently in bovine paratuberculosis.331 The contentious role of γδ T cells in adaptive immunity will be further discussed below.

Specific adaptive immune responses:

Studies over many years have demonstrated that effective resistance to progressive infection and disease due to *M. a. paratuberculosis* is associated with cell mediated rather than humoral immunity and local rather than systemic responses appear to be of most importance in the early stages of infection.64 Sheep displaying strong DTH 3 months after infection had lower viable counts of *M. a. paratuberculosis* in intestines 2-24 months later than did those showing weak
Moreover, the macrophages from strong reactors contained mainly degenerating bacilli whereas those from weak reactors contained intact bacilli, and AFB were never observed in early lesions from sheep with strong DTH. A similar relationship had been observed when hypersensitivity was induced by vaccination. Experimentally infected cattle which subsequently had no necropsy evidence of Johne's disease were observed to shed *M. a. paratuberculosis* in faeces commencing from 3-5 months after inoculation. The recovered animals displayed consistently positive DTH whereas those which developed clinical disease had inconsistent DTH. Recent studies have also demonstrated correlation of increased levels of CMI (as measured by IFN-γ production by PBMC in response to *M. a. paratuberculosis* antigen) with reduced levels of subsequent clinical disease. IFN-γ is a Th1 cytokine and is produced mainly by CD4+ T cells in infected cattle. IFN-γ levels of non-diseased experimentally infected lambs were higher than those of lambs which developed clinical disease. Similarly in cattle, infected animals identified as resistant had higher IFN-γ levels, total lymphocyte and CD8+ cell counts than susceptible animals, while animals with subclinical paratuberculosis had higher IFN-γ levels than clinical cases. At the local level, higher levels of mRNA for IFN-γ were found in ileum and MLN from subclinically infected cows as compared to clinical cases. And in sheep IPX studies revealed higher densities of CD4+ T cells in paucibacillary lesions, while multibacillary lesions had lower densities of CD4+ and CD8+ cells compared with normal tissues.

Such studies provide support for the accepted paradigm that protection against mycobacterial disease is mainly due to CD4+ Th1 cells that produce IFN-γ to activate macrophages which then kill mycobacteria during phagocytosis. Cytotoxic CD8+ cells are considered to serve a subsidiary function by releasing bacteria from infected cells which can then be killed during phagocytosis by activated macrophages. CD4+ T cells are usually MHC II restricted (ie they recognise antigens only when presented by MHC II molecules on the surface of APC), and CD8+ T cells are MHC I restricted. MHC II molecules present antigens from within phagosomes in APC whereas MHC I molecules present antigens from within the cytoplasm of the APC. Thus MHC I presentation in mycobacterial infection requires leakage of mycobacterial products from their phagosomal habitat into the macrophage cytoplasm.

The classical stage 3 of tuberculosis (development of caseous necrosis associated with development of DTH and mediated by cytotoxic T cells) does not occur in paratuberculosis.
Indeed, caseation is rarely seen in Johne's disease. The downregulation of MHC class I antigen presentation that is a feature of *M. a. paratuberculosis* infection may be a reason for this. The studies into DNA vaccination of mice against *M. tuberculosis* suggest that CD8+ memory cells which produced IFN-γ and antigen specific cytotoxicity may be even more protective than CD4+ memory T cells. A protective role for CD8+ T cells in immune responses of ruminants to *M. a. paratuberculosis* has been shown in several studies. In one series of experiments depression of the MHC II restricted proliferative response of CD4+ lymphocytes to *M. a. paratuberculosis* antigens in paratuberculous cattle was associated with the presence of γδ T cells, but the ability of γδ T cells to regulate CD4+ responses was blocked by the presence of CD8+ cells. The authors concluded that the development of protective immunity against *M. a. paratuberculosis* may be dependent on the capacity of CD8+ cells to modulate the regulatory activity of γδ T populations. In a recent study in goats high levels of *M. a. paratuberculosis* responsive CD8+ lymphocytes in the circulation about 1-year post-inoculation were associated with few gut lesions at later necropsy, whereas high numbers of γδ T cells responsive to *M. a. paratuberculosis* in the circulation were associated with disseminated lesions. An IPX study in sheep, however, demonstrated reduced numbers of CD8+ T cells in both paucibacillary (presumably indicating resistance) and multibacillary lesions. And a study of lesions in goats with multibacillary disease found decreased CD4+ T cells but increased of CD8+ T cells in the lamina propria of the intestine and MLN. The authors suggest that the progression of paratuberculous lesions may be due to an ineffective host immune response attributable to the CD8+ T cell subset that "downregulates" the activity of the CD4+ T cells required for macrophage activation.

Several studies indicate a protective role for γδ T cells in tuberculosis and leprosy in man. Increased numbers γδ T cells were present in particular granulomatous reactions of leprosy and these cells proliferated *in vitro* specifically to mycobacterial antigens. After *in vitro* culture of PBMC stimulated with *M. tuberculosis*, there were higher percentages of γδ T cells from people with effective immunity as compared to those with clinical disease. These γδ T cells produced IFN-γ, GM-CSF, IL-3 and TNF-α, all cytokines which activate macrophages. However, studies with gene knockout mice (no γδ TCR) indicate that γδ T cells do not directly contribute to protection against tuberculosis or that they do so only when bacterial loads are very high. Instead, the data suggest that γδ T cells perhaps play an important role by influencing local
cellular traffic, promoting the influx of lymphocytes and monocytes, and limiting the access of inflammatory cells that do not contribute to protection but may cause tissue damage. The situation in ruminants may be quite different. Young ruminants have uniquely high levels of $\gamma\delta$ T cells at the time of greatest susceptibility to $M. a. paratuberculosis$ infection. And specific studies in subclinically infected cattle have shown that IFN-$\gamma$ in response to $M. a. paratuberculosis$ is produced mainly by CD4$^+$ T cells, lesser amounts by CD8$^+$ cells and none by $\gamma\delta$ T cells. The association of $\gamma\delta$ T cells with disseminated lesions of paratuberculosis in goats and with depression of CD4$^+$ cell proliferation in cattle has already been described. IPX studies of paratuberculous sheep demonstrated an increased relative percentage of $\gamma\delta$ T cells in the ileum of those with multibacillary lesions, but numbers were also increased in presumably resistant sheep with paucibacillary lesions.

**Progression to clinical disease**

Clinical Johne's disease should be regarded as distinct from simple infection with $M. a. paratuberculosis$. Bendixen put it well in a 1978 review: "Paratuberculosis probably belongs to the chronic infectious diseases ... in which it is the immunological reactions of the host rather than the infectivity or metabolic activity of the agent, that are responsible for the clinical pictures."

**The Th1 to Th2 shift in the immune response:**

Recently good evidence has been found that the progressive aspects of paratuberculosis may be related to a shift from a Th1, cell mediated, to a Th2, humoral, immune response. This has been well documented in both tuberculosis and leprosy in humans and more recently reviewed by Stabel for ruminant paratuberculosis. Studies have consistently demonstrated increased levels of IFN-$\gamma$ (a Th1 cytokine) in local tissues and blood from subclinically infected cows as compared to clinical cases. Lymphocytes from sheep with paucibacillary lesions proliferated more on exposure to Johnin and produced more IFN-$\gamma$ and IL-2 (Th1 cytokines) than those from control sheep, whereas sheep with multibacillary lesions had less proliferation than uninfected controls. Local differences were more pronounced than for PBMC. However, while increased antibody levels in peripheral blood are often associated with the shift to clinical disease studies have failed to demonstrate increased mRNA for Th2 cytokines locally.

The Th1/Th2 spectrum is reflected in the pathology of paratuberculosis, particularly in sheep. Sheep with the paucibacillary form are usually positive in tests for CMI (eg DTH, IFN-$\gamma$ production by PBMC, or lymphocyte proliferation), whereas those with the multibacillary form are more often positive in tests for humoral immunity. However, the relationship between
the two forms of pathology in ruminants is not yet clear. It is possible that some sheep develop multibacillary disease directly from early focal lesions, while others develop diffuse paucibacillary disease. There may even be genetic predisposition to one form or the other as has been demonstrated for the types of leprosy in man. On the other hand animals may progress from diffuse paucibacillary disease to the multibacillary form as effective Th1 immunity wanes in progressive infection. Lesions in individual sheep might even move in both directions between the two extremes. Such is the case for leprosy in man, where exacerbations and remissions of the disease (spontaneous or due to treatment) are associated with a shift between lepromatous and tuberculoid pathology, with corresponding changes in their immune responses. The difficulties in following pathology over time in particular animals (this would require repeated intestinal and MLN biopsies) mean that this question is likely to remain unanswered for some time.

Factors triggering clinical disease:
As described above, the presence of mycobacteria themselves may depress the immune response of the host. Induction of NO synthesis by *M. avium* or *M. tuberculosis* infection of macrophages has been shown to cause decreased survival of CD4+T cells, reduced granuloma formation and reduced IFN-γ levels in mice. The effects of *M. a. paratuberculosis* infection in reducing MHC antigen presentation have already been described and are particularly important in allowing *M. a. paratuberculosis* to persist and multiply within macrophages of a host with well developed specific adaptive immunity. And some recent studies in cattle suggest that an alteration in function of chronically infected macrophages may induce apoptosis of reactive T cells leading to a progressive local depletion of Th1 cells. Other studies have shown that *M. a. paratuberculosis*-specific γδ T lymphocytes exhibit cytotoxic activity against antigen-primed CD4+T cells. Thus a gradual build-up of mycobacterial infection may eventually pass some critical point beyond which effective Th1 responses can no longer be maintained.

There are also possible hormonal triggering effects, and associations of clinical paratuberculosis with reproductive status have long been recognised. In cattle clinical disease is seen often in heifers at first parturition/lactation, and in sheep mostly in lactating ewes. In vitro studies have shown that fluctuating levels of growth hormone and prolactin made cultured bovine macrophages more permissive for the multiplication of *M. a. paratuberculosis*. In rodents and man increased cortisol, vitamin D or decreased DHEAS levels have been demonstrated to inhibit Th1 type responses. There is evidence that similar mechanisms may operate for paratuberculosis in ruminants. Experimental *M. a. paratuberculosis* infections in mice indicate that
Ca and vitamin D metabolism are important modulators of infection, while high levels of vitamin D have been shown to inhibit secretion of IFN-γ by mitogen- and antigen-stimulated bovine PBMC.

Development of clinical signs:
Very little recent work has been done on this aspect of pathogenesis (ie the immediate causes of clinical signs), presumably because management of clinical Johne's disease is rarely considered. Rather, clinically affected animals are culled because of the risk they pose to the rest of the flock. Weight loss in ruminants seems mainly due to protein malabsorption and loss caused by cellular infiltration and oedema of the intestine, but clinical disease only appears when the compensatory mechanisms of the liver can no longer balance the loss. In mice carbohydrate malabsorption has also been demonstrated. Diarrhoea may ensue due to failure of fluid resorption in affected large intestine. Undoubtedly in sheep with severe diffuse multibacillary pathology there will be significant impairment to the absorptive functions of the intestine due solely to the physical changes. But more than physical factors are involved. Decreased appetite has been demonstrated to be a significant factor in the pathogenesis of cachexia in experimental paratuberculosis in mice. This effect, presumably due to the various inflammatory processes described below which may have systemic effects beyond the local intestinal environment, probably occurs also in ruminants.

Hypersensitivity reactions have been suggested to play a role in inducing clinical signs, especially diarrhoea. Some early studies on the effects of booster vaccination provided support for this contention. In both cattle and sheep revaccination of infected animals did not enhance immunity and sometimes led to clinical disease. Other studies have demonstrated the passive transfer of johnin hypersensitivity to calves using whole blood or white cells producing fever and diarrhoea resembling clinical paratuberculosis, and clinical symptoms of Johne's disease in cattle could be abrogated by immunosuppressive treatment. In addition the occurrence of large numbers of globule leukocytes and also their frequent association with myenteric ganglionic cells in cases of severe diarrhoea in infected cattle may be morphological evidence for hypersensitivity reactions in the pathogenesis of clinical disease.

Cytokine production in lesions may also play a direct role in the development of clinical signs, and is certainly involved in the development of the underlying pathological changes. As discussed above, mycobacteria stimulate cytokine production by T cells and macrophages but are also refractory to many of the intracellular bactericidal mechanisms. Their continued
presence in high numbers in paratuberculosis may lead to overproduction of pro-inflammatory cytokines, resulting in the pathological changes of chronic inflammation. This is well described for leprosy in man.\textsuperscript{246, 280} There is some evidence for the involvement of the cytokines TNF-\(\alpha\), IL-1 and IL-6, which have been associated with granuloma formation and wasting in other disease syndromes, in the development and progression of Johne's disease. In a series of \textit{in vitro} studies \textit{M. a. paratuberculosis} or its LAM induced bovine monocytes to produce increased levels of mRNA for these cytokines, and there was no difference between monocytes from infected or uninfected cattle.\textsuperscript{2, 4} Increased levels of mRNA for TNF-\(\alpha\), IL-1\(\beta\) and IL-6 have also been demonstrated in intestinal tissues from multibacillary clinical cases of ovine paratuberculosis, whereas paucibacillary sheep showed increased levels for TNF-\(\alpha\) only.\textsuperscript{11} But the picture is certainly far from clear. Mice genetically deficient in TNF receptor, died after experimental \textit{M. avium} infection with disseminated granuloma necrosis, despite having no increased bacterial growth, indicating that TNF is beneficial in regulating the inflammatory response.\textsuperscript{95} Further, \textit{in vitro} studies of murine macrophages infected with \textit{M. a. paratuberculosis} indicate that TNF-\(\alpha\) can either enhance or reduce antimycobacterial activity depending upon both the levels of TNF-\(\alpha\) and the duration of infection.\textsuperscript{301} TNF-\(\alpha\) appears to be beneficial to the host in the early stages of infection whereas in the latter stages it plays an adverse role and may contribute to the development of immunopathology.

\textit{Humoral antibodies to M. a. paratuberculosis:}

The occurrence of humoral antibodies in clinically affected animals and in particular in animals with multibacillary disease has already been discussed. These antibodies are generally regarded as being non-protective and countless field and experimental observations in ruminants support this view. Specific studies in mice have shown that susceptibility to \textit{M. avium} infection correlates with increased synthesis of specific antibacterial antibodies, and that this was not simply due to greater numbers of bacteria in susceptible strains of mice. In addition the administration of exogenous antibody rendered resistant mice susceptible, indicating that not only was antibody non-protective, it was deleterious.\textsuperscript{101} Later studies have shown clearly a genetic influence on antibody production. Strains of mice varied in their expression of primarily Th1 or Th2 cytokines after \textit{M avium} infection.\textsuperscript{234} Mice with predominantly Th1 responses produced a more limited range of antibodies and only of the IgG2a isotype, whereas those with predominantly Th2 responses produced a mixed IgG1 and IgG2a response to a wider range of mycobacterial antigens. There is similar variation in antibody production in ruminants. The observed antibody increases in clinically affected animals are directed only to certain antigens, in particular to those found in PPD preparations. And only the IgG1 isoforms are increased.\textsuperscript{104}
PPD antigens are secreted protein antigens that with heavy bacterial loads in infected tissues are released to the circulation and can stimulate B cells remote from the lesions. Other antigens such as the heat shock proteins or LAM are cell wall associated and likely to have more local effects; antibodies to these were not shown to increase with the shift to clinical disease. In fact levels were decreased compared to those in subclinically affected animals. Measurement of such alternate antibody may even allow earlier serological detection of *M. a. paratuberculosis* infection than has been the case to date.

But despite the undoubted importance of cell mediated immunity, and the lack of a protective function for antibody, a role for B cells in protective immunity to paratuberculosis is possible. B cells can play a role as antigen-presenting cells and in granuloma formation, and B cell deficient mice experimentally infected with *M. tuberculosis* developed more severe infections than normal mice. B cells from peripheral blood of cattle with subclinical paratuberculosis proliferated vigorously in response to *M. a. paratuberculosis* antigen, whereas those from animals with clinical disease, or from uninfected animals did not, suggesting some role for B cells in resistance to clinical paratuberculosis. The authors concluded that B-cell proliferation is a sensitive indicator of subclinical Johne's disease and that the immunologic mechanisms responsible for this may be significant in the eventual progression from subclinical to clinical Johne's disease in cattle. Interestingly, clinically affected cattle had abnormally high proportions of B cells in peripheral blood, suggesting that differentiated B cells (plasma cells) in the tissues are producing antibody, while the large number of B cells in the blood are specifically unresponsive to *M. a. paratuberculosis* antigens.

**Recovery**

Recovery from *M. a. paratuberculosis* infection or even from clinical Johne's disease is rarely considered, since most management strategies involve culling of infected animals. But there is ample evidence that some degree of recovery may occur at all stages of the pathogenesis of the disease. Certainly many animals in highly contaminated environments are exposed to high levels of *M. a. paratuberculosis* infection throughout their lives yet fail to succumb to the disease. And the association of good CMI responses with resistance to clinical disease in experimentally infected animals has already been discussed. An early report describes sustained recovery from clinical infection in a cow, and there are several reports of experimental infections or field observations in which cattle regularly excreted *M. a. paratuberculosis* but later were negative on repeated faecal examination. These latter represent recovery from subclinical “shedder” status although such animals may continue to be silently infected. Several
animals in one study had no evidence for infection at subsequent necropsy, so may have fully recovered. Experimentally infected sheep were mostly culture positive from intestinal tissues one to two months after infection, but few were positive when examined nine months later, indicating recovery in some sheep, presumably as specific adaptive immunity developed. In related studies *M. a. paratuberculosis* was cultured from intestinal tissues in the first 12 to 36 hours after infection, but not after one week, while some sheep were again positive after some months. This may indicate recovery during the earliest stages of infection where innate immune responses deal with much of the infectious load. In one of the few prospective studies employing repeated intestinal and MLN biopsy to monitor individual animals over time, recovery was demonstrated in 2 of 5 experimentally infected sheep (no bacteriological or histopathological signs of infection at necropsy 27 months post infection). A further sheep had mild microscopic lesions but was culture negative. All sheep had lesions and positive cultures from biopsies 5 and 11 months post infection. But, of course, whether or not complete recovery is common or even occurs may always remain an academic question. Persistent, isolated, focal, possibly infective lesions may readily be missed by even the most thorough necropsy!
Chapter 2. Development of methods for the enumeration of ovine strains of Mycobacterium avium subsp. paratuberculosis

Summary
Alternative methods to endpoint titration in liquid media for the determination of viable numbers of Australian ovine strains of M. a. paratuberculosis were evaluated. Direct counts, spectrophotometry and colony counts on solid media gave inconsistent results. A simple relationship between the time taken for the cumulative growth indices in Bactec cultures to reach 1000 (CGI1000) and numbers of viable organisms in inocula was established. For an isolate characterised in an MPN trial, CGI1000 provided an estimate of viable numbers within narrow prediction bands. The relationship was also applicable for the enumeration of uncharacterised isolates from a variety of sources with prediction bands of +/- 1.5 to 2 logs. Storage of cultured suspensions at -80 °C was shown to result in minimal loss of viable numbers. Routine decontamination procedures necessary for the isolation of ovine strains of M. a. paratuberculosis from faeces and tissues were shown to result in a reduction of numbers of viable organisms of 1 to 2 logs. Together, these findings will facilitate future experimental infections and the efficient determination of viable numbers of ovine strains of M. a. paratuberculosis in clinical and environmental samples.

General introduction
In the study of Johne's disease there is a need to quantify the numbers of Mycobacterium avium subsp. paratuberculosis (M. a. paratuberculosis) in experimental inocula for comparison between different trials, to determine the level of infection in animal tissues or excretions, and levels of environmental contamination. Mycobacteria in general, because of their slow growth and tendency to clump have been difficult to quantify using routine bacteriological methods. Fenner in 195199 described and validated a simple surface drop plate count technique for the enumeration of viable tubercle bacilli. Similar techniques, using appropriate media would be applicable to M. a. paratuberculosis. Indeed, colony counts have been used for 40 years to enumerate viable M. a. paratuberculosis of bovine origin.41 59 325 380 Microscopy, using either unstained bacterial suspensions in counting chambers, or stained smears has long been used in the enumeration of mycobacteria.99 Such techniques have the advantage of rapid results and do not require that organisms can be grown in culture. However they do not differentiate between viable and non-viable organisms and are of limited use in tissues or faeces, or where small numbers of organisms are present. These techniques are also non-specific, as other AFB are not infrequent in clinical samples. The opacity (measured by nephelometry or
spectrophotometry) of suspensions of cultured bovine strains of *M. a. paratuberculosis* has also been used as a rapid technique to estimate numbers.\textsuperscript{42}

However, experimental inocula are still often recorded only as weight of organisms administered,\textsuperscript{209} or even as weight of infected mucosa\textsuperscript{314} and comparison of doses between different investigations is often difficult, if not impossible. However, comparisons using calculations based on size and specific gravity of the bacilli are possible. *M. a. paratuberculosis* is roughly a cylinder 1\(\mu\)m long and 0.5\(\mu\)m diameter, with similar specific gravity to *Mycobacterium tuberculosis* (1.045), which implies a weight of 2 x 10\(^{-10}\)mg per organism, ie 5 x 10\(^{9}\) organisms per mg (dry weight).\textsuperscript{194} Brotherston *et al.*\textsuperscript{42} reported that 2mg wet weight of colonies of *M. a. paratuberculosis* from solid media contained about 10\(^8\) to 10\(^9\) viable units. Microscopically these units were “single bacilli or small clumps with rarely more than 10 bacilli per clump”. Assuming wet weight is similar to that for *Mycobacterium tuberculosis* at about 85% water,\textsuperscript{258} Brotherston’s figures converted to dry weight are about 3.3 x 10\(^8\) to 3.3 x 10\(^9\) units per mg, a little less than the theoretical calculation above. When one allows about a five-fold underestimate because Brotherston’s figures are for colony forming units (CFU), then the results (1.6 x 10\(^9\) to 1.6 X 10\(^{10}\)) are in very close agreement to the theoretical calculations. Compare to Petroff’s detailed quantitative observations for the larger *M. tuberculosis* of 3 X 10\(^8\) / mg dry weight, or 4.5 X 10\(^7\) /mg wet weight.\textsuperscript{258} Taken together the above findings and calculations suggest rough working figures for *M. a. paratuberculosis* of about 5 x 10\(^9\) organisms/mg dry weight or 7.5 x 10\(^8\)/mg wet weight. These figures can be used for rough comparisons between some reported investigations, and sometimes for alternative interpretations of claimed dose rates. For example, material harvested directly from infected mucous membrane of a steer was claimed to contain about 10\(^6\) organisms/mg (dry weight).\textsuperscript{159} This was probably a ten-fold underestimate since it appears that Petroff’s figures\textsuperscript{258} for the larger *M. tuberculosis* were used to estimate numbers. Juste *et al.*\textsuperscript{150} challenged lambs with 150 mg wet weight of a bovine isolate, suggesting with no description of methods that the inoculum contained 1.36x10\(^6\) organisms, whereas the above calculations yield a figure in excess of 10\(^{10}\) organisms.

There are further complications in the enumeration of *M. a. paratuberculosis* from clinical samples (eg tissue collected at necropsy, or faeces). Decontamination protocols are required to prevent contamination of culture media by other gut flora and concentration techniques are used to extract organisms from gram quantities of samples. Many different techniques are used by laboratories for these cultures and much effort has gone into optimising the balance.
between too little decontamination leading to contaminated cultures and too rigorous decontamination leading to false negative cultures. But regardless of which method is used all protocols will reduce the numbers of *M. a. paratuberculosis* isolated from clinical samples. Several authors have measured or estimated this loss to be about 1-2 logs, using different culture techniques. Mokresh et al reported a reduction by about 2 logs after exposure to 0.75% hexadecylpyridinium chloride (HPC) for 18 hours, although other workers have reported no effect of HPC for up to 5 days.

The usual ovine strains of *M. a. paratuberculosis* present particular problems. Until recently they were not reliably grown *in vitro* at all. Reliable growth is now possible in liquid modified Bactec 12B medium. Solid media (modified Middlebrook 7H10 and 7H11 agars) were also demonstrated to support growth but may be less sensitive. Thus experimental work in sheep has frequently been done with cultured *M. a. paratuberculosis* of bovine origin. In fact much of the early work in sheep was done as a model for the bovine disease. In Australia sheep are rarely infected with the bovine strains, and meaningful studies into the pathogenesis of Johne’s disease in sheep or the survival of the organism in the environment may require the use of *M. a. paratuberculosis* of ovine origin. We have used end-point titration (also known as most probable number (MPN) estimation) in liquid modified Bactec 12B medium to quantify *M. a. paratuberculosis* (ovine strain) in sheep faeces. This technique is time consuming (at least 12 weeks to be sure of negative growth in tubes at high dilutions), very expensive for materials, subject to error if significant clumping of organisms is present, and underestimates numbers by an unknown extent due to the decontamination procedures.

Lambrecht et al described a mathematical model for estimating from cumulative growth index (CGI) the numbers of viable *M. a. paratuberculosis* of a laboratory-adapted bovine strain inoculated into a single Bactec vial. They standardised their estimates against plate counts, and stored aliquots of suspensions at -70 °C, apparently without loss of viable numbers. Their method was used to quantify *M. a. paratuberculosis* excretion in faeces of subclinically affected cattle after sample decontamination, and subsequently to quantify *M. a. paratuberculosis* infection in macrophages. Bactec growth index (GI) measurements (at 24 hours after inoculation) were also shown to correlate well with conventional plate counts for *Mycobacterium avium* from infected macrophage cultures, and from mouse tissues after experimental infection. Collins et al reported correlation of detection times for *M. a. paratuberculosis* in radiometric culture for bovine faecal specimens with severity of disease, and by inference with
numbers of organisms (13.4 +/- 5.9 days with smear-positive specimens, 27.9 +/- 8.7 days from subclinically infected cows with moderate pathology, and 38.7 +/- 3.8 days from subclinically infected cows with low-grade pathology). Direct radiometric measurement offers several potential advantages over both end-point titrations and plate counts for the enumeration of the ovine strains of *M. a. paratuberculosis*. These include cost, time, being less affected by clumping, and not requiring reliable growth on solid media.

The aim of this study was to:

1. Evaluate the use of direct counts, spectrophotometry and colony counts on solid media as alternatives to MPN estimation for the enumeration of *M. a. paratuberculosis* (ovine strain).

2. To assess the effect of 0.1% Tween-80 to reduce clumping in the serial dilutions used to provide MPN estimates for *M. a. paratuberculosis* (ovine strain).

3. To investigate the use of cumulative growth indices in Bactec cultures for the enumeration of *M. a. paratuberculosis* (ovine strain).

4. To assess the effect of storage at 4 °C, -20 °C or -80 °C on the survival of cultured *M. a. paratuberculosis* in phosphate buffered saline with 0.1% v/v Tween-80 (PBST) suspensions.

5. To quantify the effect of decontamination protocols on the numbers of *M. a. paratuberculosis* of ovine origin isolated from tissues and faeces.

**General methods**

**Preparation of suspensions from cultures:**

Modified 7H10 slopes were inoculated with 100 μL of broth from *M. a. paratuberculosis* cultures in Bactec vials which had achieved a weekly growth index of 999. Colonies were collected from the surface of these slopes after 6 weeks incubation (unless otherwise specified) by washing with 200μL phosphate buffered saline (PBS) or PBS with 0.1% Tween-80 (PBST) per slope using a sterile plastic loop to produce a thick suspension (termed undiluted suspension) which was thoroughly mixed by vortexing. Serial 10-fold dilutions were made in PBS or PBST.
PROCESSING OF TISSUES FOR BACTEC CULTURE:

Approximately 2g of tissue was trimmed of fat and fibrous tissue and homogenised for 30 s in 2mL of sterile normal saline in a blender. After adding 25 mL of 0.75% HPC (Sigma Chemical Co., St Louis, Mo), the homogenates were left standing at room temperature for 72 hours. In routine processing 100 μL of sediment was carefully removed from near the bottom of the tube with a 26 g needle attached to a tuberculin syringe for subsequent inoculation into a Bactec vial. In the alternate centrifugation method the tube was centrifuged at 900 X g for 30 mins. The pellet was resuspended in 1mL of 0.75% HPC by vigorous agitation and vortexing. 100 μL of the resulting suspension was used to inoculate a Bactec vial. For MPN estimations and plate counts, 100 μL of the suspension was added to 900 μL PBST to prepare a 10⁻¹ dilution. This was vortexed for 1 min, passed 10 times through a 26 g needle, and subsequent 10 fold dilutions were then prepared in PBST, vortexing for one minute between each dilution step.

PROCESSING OF SOIL AND FAECAL SAMPLES FOR BACTEC CULTURE:

Preparation was by the method in routine use at the Elizabeth Macarthur Agricultural Institute (EMAI), which is based on the double incubation method of Whitlock and Rosenberger. Briefly, 2g of faeces or soil was placed in a 15mL polypropylene tube and broken up in 10-12 mL of sterile normal saline using a swab stick. After mixing the tube was allowed to stand for 30 min at room temperature. A 5mL aliquot of the surface fluid was transferred to a 35mL polystyrene tube containing 25mL of 0.9% HPC in half-strength brain heart infusion broth (BHI, Oxoid, Basingstoke, England) and allowed to stand at 37 °C for 24 h. The tube was centrifuged at 900 X g for 30 mins. The pellet was resuspended in 1mL of sterile water with vancomycin (100 μg/mL), nalidixic acid (100 μg/mL) and amphotericin B (50 μg/mL) (VAN) and incubated for 72 h at 37 °C. Sediment was then resuspended by vigorous agitation, and 100 μL of the resulting suspension used to inoculate a Bactec vial. For MPN estimations serial 10-fold dilutions were made in PBS or PBST.

CULTURE IN LIQUID MEDIA:

100 μL of inoculum was added to each Bactec vial, and the vials were incubated at 37 °C for up to 12 weeks. The modified Bactec 12B radiometric medium consisted of 4mL enriched Middlebrook 7H9 medium (Bactec 12B; Becton Dickinson, Sparks, Md.) with 200μL PANTA PLUS (Becton Dickinson), 1 mL egg yolk, 5μg of mycobactin J (Allied Monitor Inc., Fayette, Mo.) and 0.7 mL of water. Growth indices (GI) were measured weekly with an automatic ion
chamber (Bactec 460; Johnston Laboratories, Towson, Md). PCR for IS900 and REA were performed on material from GI positive vials to confirm that the observed GI were due to *M. a. paratuberculosis*.

**MPN estimates:**

Five 100μL replicates of each of up to ten serial tenfold dilutions in PBS or PBST were inoculated into Bactec vials. The MPN estimate and 95% confidence limits were read from published tables using the culture results (number of GI positive samples from the five replicate vials) for three appropriate sequential dilutions. The number per mL of *M. a. paratuberculosis* in undiluted suspension was calculated by allowing for the dilution factor and the inoculum volume (number/mL = MPN value from tables x 1/dilution x 1/0.1). IS900 PCR was performed on material from Bactec vials at the critical end-point dilutions to confirm that the observed GI were due to *M. a. paratuberculosis*.

**Acid fast stained smears:**

Smears were prepared from suspensions, tissues or faeces, dried at 65 °C, and stained by a Ziehl-Neelsen (ZN) technique. Counts of acid-fast organisms (AFB) were done on smears from some selected suspensions. AFB in 5 oil-immersion fields (X 1000) were counted. Counts of “bacterial units” (where a clump of AFB is counted as a single unit) were compared with counts of single cells (where every individual AFB is counted).

**Direct counts using Thoma counting chamber:**

These were performed only on suspensions prepared from cultured *M. a. paratuberculosis* because particulate matter in other types of suspensions precluded the use of this technique. 4 μL of an appropriate dilution of bacterial suspensions in PBS, PBS with 0.1% v/v Tween-20 or PBST was added to the counting chamber after vortexing for 30 s. The dilution was chosen to give about 3-10 organisms per small square in the counting chamber. After loading, the diluted suspensions were allowed to settle for at least 2 minutes. Four groups of 16 small squares were counted under dark field illumination at 200X magnification, focusing up and down to check that all organisms were counted. The number of bacteria in the undiluted suspension was given by: average count in 64 squares x dilution factor x 3.125 x 10^5. Counts were done on duplicate 4μL aliquots.
SPECTROPHOTOMETRIC ANALYSIS:
Serial two-fold dilutions in PBS of cultured *M. a. paratuberculosis* starting from $10^{-1}$ or $10^{-2}$ dilutions were used. 1.0 mL of the diluted suspension was added to a disposable cuvette and optical density (OD) at 600nm was read in a Hitachi U-1100 spectrophotometer.

COLONY COUNTS ON SOLID MEDIA (PLATE COUNTS):
Culture plates consisted of 13 mL agar in sterile polystyrene airtight screw-top jars of 60mL capacity and 40mm diameter (Labserv, Auckland, NZ). Plates were poured on a level platform and dried for approximately one hour. The agar was modified Middlebrook 7H10 medium (briefly, Middlebrook 7H10 agar (Difco) enriched with 4.0 g bovine albumin fraction V, 1.6 g dextrose, 5 mL glycerol, 2.4 mg catalase, 200 mL egg yolk, 1.0 mg mycobactin J and 40 mL PANTA PLUS per litre). Methylene blue (0.02% w/v) (MB) was added in some trials to aid visualisation of small colonies. The method of inoculation was based upon Miles and Misra’s surface drop count, except that only one drop per plate was used. One drop of inoculum (10 or 20 µL from Gilson calibrated pipette) was added to the centre of each plate from a height of approximately 20 mm. Four or five replicate plates were used for each dilution. Up to seven serial ten-fold dilutions in PBS or PBST were used for each enumeration trial. Plates were incubated at 37 °C for up to 26 weeks and examined weekly without opening until colonies were visible. Colony counts were then performed on the opened plates under a dissecting microscope, and repeated in subsequent weeks, until the count was stable or falling due to coalescence of colonies. The dilution used for counting was that giving the highest number of colonies per drop which could be readily counted. This was usually between 10 and 100 colonies per drop.

2.1. Evaluation of direct counts, spectrophotometry and colony counts on solid media as alternatives to MPN estimation for the enumeration of *M. a. paratuberculosis* (ovine strain).

*Introduction and aims*
Currently MPN estimations are the only tool available to assess numbers of viable *M. a. paratuberculosis* of Australian ovine strains. The technique is time consuming and expensive. This series of trials investigated the utility of direct counts, spectrophotometry and plate counts as possible simple, inexpensive or rapid alternatives to MPN estimations. When applied to cultures of *M. a. paratuberculosis* such techniques could facilitate the standardisation of experimental inocula. Because all previous reports referring to enumeration of *M. a. paratuberculosis*, and almost all previous experimental infections in sheep, have used strains with
growth characteristics of the bovine strains, a bovine strain was included for comparative purposes in this study.

Methods

Enumeration Trials:

Trial 1: Undiluted suspensions and subsequent dilutions were prepared in PBS from slopes (pass 2) prepared from Bactec cultures of *M. a. paratuberculosis* isolated from the faeces of 19 sheep with clinical paratuberculosis. Identity was confirmed by IS900 PCR and subculture to solid media as previously described. The bacterial suspensions were pooled into three groups on the basis of ages of the slopes as follows: Trial 1a, 5-7 weeks (5 slopes); Trial 1b, 8-9 weeks (6 slopes); Trial 1c, 10-13 weeks (8 slopes). MPN estimations (10^{-4} to 10^{-10} dilutions), ZN smears, direct counts, spectrophotometry and plate counts (10^{-7} to 10^{8} dilutions, 20\mu L inoculum, 5 replicates per dilution) were performed on the suspensions for each trial.

Trial 2: Trial 2 was essentially a repeat of Trial 1a, to see whether the results for 6 week old slopes were repeatable. Undiluted suspension was prepared from slopes (pass 4) prepared from a Bactec culture used in Trial 1a MPN estimation. MPN estimation (10^{-6} to 10^{-12} dilutions), ZN smears and counts, direct counts, spectrophotometry and plate counts (10^{-2} to 10^{8} dilutions, 10\mu L inoculum, 5 replicates per dilution) were performed.

Trials 3, 4 and 5: Undiluted suspensions and subsequent dilutions were prepared in PBS from slopes (pass 5) prepared from fresh Bactec vials inoculated from the same vial used to produce slopes for Trial 2. These suspensions were used for experimental inoculation of sheep in a pathogenesis study (Chapter 3). MPN estimations (10^{-6} to 10^{-11} dilutions), ZN smears, direct counts, spectrophotometry and plate counts (10^{-2} to 10^{8} dilutions, 10\mu L inoculum, 4 replicates per dilution, 2 with MB and 2 without) were performed.

Trial 6: Suspensions and subsequent dilutions were prepared in PBST, and each suspension was prepared from a single slope. The 10^{-7} dilution was passed 10 times through a 26 g needle and filtered through a 8\mu m filter in an attempt to produce suspensions with minimal clumping of organisms. Trial 6a used undiluted suspension from a bovine laboratory-adapted strain (316V). Trial 6b used undiluted suspension prepared from slopes (pass 5) as in Trials 3-5. Trial 6d used undiluted suspension from a 10 wk old slope (pass 2) of *M. a. paratuberculosis* isolated from the ileum of a sheep experimentally infected with *M. a. paratuberculosis* (Sheep 40, Chapter 3). MPN estimations (10^{-3} to 10^{9} dilutions, 6a; 10^{-2} to 10^{8} dilutions, 6b; 10^{-1} to 10^{-7} dilutions, 6d), ZN smears and counts, direct counts and plate counts (10^{-2} to 10^{8} dilutions, 10\mu L inoculum, 4 replicates per dilution, all with MB) were performed. For comparative purposes the results were expressed in terms of the “theoretical” undiluted suspension (10 times the concentration...
of the $10^{-1}$ dilution) which would contain fewer organisms than the actual undiluted suspension because some would have been removed by the filtration. In these trials GI readings were taken every 1-3 days (see Section 3).

**Trial 7:** Suspensions were prepared from terminal ileal samples from sheep known to be infected. These samples had been stored at -80 °C for up to 7 months. Samples used in Trials 7a and 7b were from 2 different sheep with multibacillary Johne's disease, those used in 7c and 7d were from paucibacillary cases, and those in 7e to 7h were from sheep with no lesions that had been experimentally inoculated with *M. a. paratuberculosis*. MPN estimations (undiluted to $10^{-10}$ dilution, 7a,b; undiluted to $10^{-7}$ dilution, 7c,d; undiluted to $10^{-4}$ dilution, 7e,f,g,h), ZN smears and plate counts (10 μL inoculum, 4 plates per dilution, all with MB; $10^{-2}$ to $10^{-8}$ dilutions, 7a,b; undiluted to $10^{-6}$ dilution, 7c,d; undiluted to $10^{-4}$ dilution, 7e,f,g,h) were performed. The dilutions used were estimated from ZN smears and histologic examinations (7a to d), or from known culture results of replicate samples (7e to h). The range of dilutions for inoculation of Bactec vials was selected to use the minimum number of dilutions, while still having some vials beyond the end-point dilution. IS900 PCR and REA was performed on material from Bactec cultures at the highest dilutions with growth to confirm that the observed growth indices were due to *M. a. paratuberculosis*. GI readings for these trials were taken every 1-3 days (see Section 3).

**Results**

**PREPARATION OF SUSPENSIONS FROM CULTURES:**

The ovine strain of *M. a. paratuberculosis* emulsified easily in PBS without the addition of wetting agents which are commonly needed in work with other mycobacteria. However, the bovine strain 361V used in Trial 6a could not be suspended at all in PBS without Tween-80. Undiluted suspensions were usually white and opaque to the naked eye, with a slightly creamy consistency, except suspension from 6d which was more translucent with a milky consistency. It was also noted at the time of harvesting the colonies from the slope used to prepare suspension for 6d that growth was less than expected.

**MPN ESTIMATIONS:**

Results are given in Figures 2.1.1. (ex cultures) and 2.1.2 (ex tissues). The MPN results given for Trials 6b and 7g are minimum values, since growth was obtained in all vials at the highest dilution inoculated. No growth was observed in any material from Trial 7d.
Figure 2.1.1. MPN estimations, direct counts and plate counts of *M. a. paratuberculosis* in suspensions obtained from cultures

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Direct count</th>
<th>MPN estimation</th>
<th>Plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>5.5</td>
<td>6.0</td>
<td>6.5</td>
</tr>
<tr>
<td>1b</td>
<td>5.7</td>
<td>6.2</td>
<td>6.7</td>
</tr>
<tr>
<td>1c</td>
<td>5.9</td>
<td>6.4</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>6.1</td>
<td>6.6</td>
<td>7.1</td>
</tr>
<tr>
<td>3</td>
<td>6.3</td>
<td>6.8</td>
<td>7.3</td>
</tr>
<tr>
<td>4</td>
<td>6.5</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td>5</td>
<td>6.7</td>
<td>7.2</td>
<td>7.7</td>
</tr>
<tr>
<td>6b*</td>
<td>6.9</td>
<td>7.3</td>
<td>8.0</td>
</tr>
<tr>
<td>6d*</td>
<td>7.1</td>
<td>7.5</td>
<td>8.1</td>
</tr>
<tr>
<td>6a*</td>
<td>7.3</td>
<td>7.8</td>
<td>8.3</td>
</tr>
</tbody>
</table>

All trials are for ovine strains, except 6a (bovine laboratory adapted strain).

* Suspensions treated to remove clumps, results expressed in terms of "theoretical" undiluted suspension

Error bars for MPN estimations are 95% confidence intervals provided by MPN tables. Error bars for direct counts and plate counts are 2 x standard deviation of replicate counts.

# Error range exceeds scale of graph because of large variation in replicate counts

Figure 2.1.2. MPN estimations and plate counts of *M. a. paratuberculosis* in suspensions obtained from decontamination of infected sheep tissues

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Log10 number of organisms in undiluted suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>8.0</td>
</tr>
<tr>
<td>7b</td>
<td>9.0</td>
</tr>
<tr>
<td>7c</td>
<td>8.5</td>
</tr>
<tr>
<td>7e</td>
<td>7.5</td>
</tr>
<tr>
<td>7f</td>
<td>6.5</td>
</tr>
<tr>
<td>7g</td>
<td>5.5</td>
</tr>
<tr>
<td>7h</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* No growth at any dilution on solid media

Error bars for MPN estimations are 95% confidence intervals provided by MPN tables.

# Large variation in replicate counts
DIRECT COUNTS:

Results are included in Figure 2.1.1. Direct counts were higher than, and outside the 95% confidence limits of the MPN estimations in all trials except 1a, 6b and 6a. The direct counts were similar across all trials (9.71 to 10.32), except Trial 6d which had a count of only 8.67. The variation between replicate counts was negligible in most trials. Dilutions between 1:100 and 1:200 were suitable for use in the counting chamber. A subjective visual assessment of the degree of clumping was made at the time of counting. Suspensions in PBS from Trial 1 were initially prepared and examined with and without Tween-20. For *M. a. paratuberculosis* of ovine origin the majority of bacterial cells were present as apparently single cells, with occasional clumps of two or three organisms regardless of the method of preparation. Occasional larger “clumps”, which were seen only in PBS suspensions prepared without Tween-20, consisted of mainly amorphous material with just a few bacteria embedded and were interpreted as media remnants. However, their presence precluded meaningful counting by affecting the random distribution of organisms on the grid of the counting chamber. Consequently the direct counts reported for Trials 1-5 are all for suspensions diluted in PBS with Tween-20. Filtering had no effect on the appearance of suspensions of the ovine strains (Trials 6b and 6d) of *M. a. paratuberculosis* in the counting chamber. In contrast, unfiltered PBST suspensions of the bovine strain had many large clumps of bacilli. Filtering reduced numbers in the bovine suspension 6a from 10.33 to 9.95 (an almost 3 fold reduction). In the ovine suspensions there was little difference between unfiltered and filtered suspensions (9.79 to 9.63 for 6b and 8.60 to 8.70 for 6d).

ZN STAINED SMEARS:

ZN stained smears were examined and counts done from selected suspensions. Clumping was more prominent than in the wet suspensions, possibly because the organisms aggregated as the smear dried. For the ovine strains there was little difference in the counts from ZN smears between PBS suspensions prepared with or without Tween-20. Suspensions prepared in PBS alone consistently had bacilli/”bacterial units” ratios of about 1.6. Suspensions prepared with the addition of Tween-20 had ratios between 1.3 and 1.7. Suspensions of ovine strains prepared in PBST, passed through a fine gauge needle and filtered had ratios between 1.1 and 1.2, indicating a reduction in the number of clumps which was not obvious in the subjective assessment of suspensions in the counting chamber. Acid fast organisms in ZN smears made from unfiltered PBST suspensions of the bovine strain could not be counted due to large clumps with densely packed organisms. The filtered suspension had a bacilli/”bacterial units”
ratio of 1.3. Stained smears from the tissues used for 7a and b had abundant AFB, often in clumps. Only occasional single organisms were seen in 7c and 7d, and no organisms were visible in tissue smears from 7e, f, g and h.

Spectrophotometry:

Spectrophotometric results for Trials 1 to 5 compared to numbers determined from the direct count are shown in Figure 2.1.3. For each suspension preparation there was an approximately linear relationship between concentration of organisms in each dilution and the OD. At any given OD between 0.1 and 0.5 there was a four to six-fold variation in numbers of organisms determined from the direct count across the suspensions assessed. The technique was relatively insensitive, and could not be used for suspensions containing less than about $10^7$ organisms/mL.

**Figure 2.1.3. Spectrophotometry results compared to direct counts for suspensions prepared from cultured M. a. paratuberculosis**

Colony growth and counts on solid medium:

Results for plate counts are included in Figures 2.1.1 (ex cultures) and 2.1.2 (ex tissues). Plate counts were lower than, and outside the 95% confidence limits of the MPN estimations in all trials except 1a, 2, 3, 6a, 6d and 7f. There was considerable variation in the counts obtained from replicate plates in several trials, notably those prepared from tissue suspensions. The presence or absence of MB did not affect the numbers of colonies on plates (data not shown). All plates were incubated for 6 months but no further change beyond the appearance at 16 weeks incubation was noted. Growth was apparent on the plates from the lowest dilutions of
suspensions from cultures by 4 weeks in some cases. On plates from higher dilutions growth was never visible before 6 weeks and on some plates no colonies were visible until 12 weeks of incubation. Counts were usually done between weeks 9 and 16. The appearance of the culture plates is shown in Figure 2.1.4. Beyond about 12 weeks of incubation on culture plates with dense growth, there was often a marked, apparently random variation in colony size, frequently with some larger colonies spread amongst a lawn of smaller ones. This effect is shown in Figure 2.1.5. Addition of MB to the medium greatly aided the visualisation of colonies, which took up the dye and appeared dark blue. Figure 2.4.6 shows the appearance of colonies at a range of dilutions on plates with MB. Growth was not observed on any plate cultures from suspensions obtained from infected tissues until week 10 and counts were attempted at week 13. Colonies were tiny, only visible because they had taken up methylene blue, and could only be counted using the dissecting microscope and even then it was difficult to discern whether all little blue dots were really colonies. No growth at all was obtained on solid medium from 7d nor from 7a and b, despite these latter two tissues having the highest numbers of organisms in smears and MPN estimates.

**Figure 2.1.4. Culture plates used for enumeration of M. a. paratuberculosis**
(plates with and without methylene blue are shown)
Discussion

In Trials 1a and 6b there was no difference between the direct counts and the MPN estimations, suggesting that all observed organisms were viable in the liquid culture medium and that any clumping of the organisms had a similar effect in both counting systems. However, direct counts were up to 1.9 logs higher than MPN estimates in other trials with
ovine strains of *M. a. paratuberculosis*, which would limit the use of direct counts as a consistent estimate of the likely MPN results.

For spectrophotometry to be useful the OD should correlate well with the direct counts of organisms. There was a four to six-fold variation (0.6 to 0.8 logs) in absolute numbers of organisms as determined by direct counts of suspensions from different trials at the same OD. Remnants of the solid medium were probably contributing to the OD and it is highly likely that the amount of carryover of egg yolk (or other constituents of the medium) varied between different undiluted suspension preparations. Variations in size distribution of bacilli or degree of clumping in cultures of differing ages or passage levels may also have had an effect. Spectrophotometry needed a volume of one mL to read which was wasteful of inocula. The technique did allow a rapid estimate of the direct count, but probably no better than “eyeballing” the neat suspension, as was possible with Trial 6d. It was not used in further work. Direct counts gave more information (including allowing some assessment of clumping) and needed a smaller sample.

The colonies of the ovine strain of *M. a. paratuberculosis* were difficult to count on the modified Middlebrook 7H10 slopes in routine use in the EMAI laboratory. Colonies were usually smaller and less distinct than those of the bovine strain. The plate technique described here allowed a drop method to be used which provided a good spread of colonies and also facilitated the use of a dissecting microscope for counting. This work confirmed previous suggestions that solid media may be less sensitive in detecting the ovine strains of *M. a. paratuberculosis* than liquid media.³⁶⁸ The plate technique described here may be even less sensitive than the usual 7H10 slopes, with 2 of 7 confirmed positive tissue samples failing to grow at all on the plates, but a specific comparison between slopes and plates was not undertaken. The unreliable growth in this system precluded its use as a routine enumeration technique for *M. a. paratuberculosis* of ovine origin. Reasons for this unreliable growth are unclear. The lids of the plates had a tendency to loosen with incubation, sometimes leading to desiccation of the medium and this may have affected growth in some samples. Similarly, polystyrene may be a less suitable material than polypropylene (used for the routine 7H10 slopes) for extended incubation periods. An interesting observation was that colonies growing close together were often larger and were visible sooner than isolated colonies. On plates with heavy growth colonies were often visible as early as 4 weeks post inoculation whereas isolated colonies were not discernible, even using a dissecting microscope, until 6 or more weeks. These observations suggest that *M.
a. paratuberculosis growing on solid medium may produce some growth factor which is lost to isolated colonies by diffusion, but is available when colonies are close together, and that the medium may be only marginally suitable for the growth of the organism. On plates with very dense growth an effect of crowding on reducing final colony size was also observed.

Across Trials 1a, 1b and 1c there was a trend for MPN estimates of suspensions prepared from older slopes to be lower (compared to direct counts) than those from younger slopes, and for plate counts to be lower again. Such results are consistent with expectations. Bacteria from older slopes are less likely to be actively dividing, indeed some may even be non-viable. Marginally viable organisms may be able to recover and grow in the liquid media used for the MPN estimations, but unable to do so on the less favourable solid media. This trend, however, disappears if one considers also the results of Trials 2-5, which were all from 6 week old slopes, but had relative MPN estimates even lower than those of Trials 1b and 1c from older slopes. A possible confounding factor in the interpretation of any effect of the age of the source slope is the passage level of the M. a. paratuberculosis used. One might expect that with increasing passage level some adaptation to artificial culture might occur and viability in such culture would be increased. This did not appear to be the case, with viable counts in Trial 2 (pass 4) less than in Trial 1a (pass 2), and those in Trials 3, 4 and 5 (pass 5) lower again. An alternative explanation to a decrease in viability in artificial media with either age of slope or increasing passage level is that the degree of clumping of organisms was increased, thereby decreasing the apparent viable counts. Clumping would have most effect in the more dilute PBS suspensions at the critical dilutions for MPN estimates, but there are too few organisms in such dilute suspensions for direct examination. The preparation of suspensions in Trials 6a, 6b and 6d was designed to minimise the occurrence of clumps, and in 6a and 6b (both from 6 week old slopes) there was no significant difference between the direct counts and MPN estimates. The effects of clumping are examined specifically in Section 2 of this chapter. Different batches of both liquid and solid media were used for Trials (1a-c), 2, (3, 4, 5), (6a, 6b, 6d) and (7a-h). Egg yolk, which is currently an essential component of these media, is a natural product and the chemical composition may vary between batches (perhaps depending on the source of eggs or on length of storage), possibly affecting the viability or growth of M. a. paratuberculosis in cultures. Any such effect would further confound interpretation of the data.

It is interesting to note that the bovine strain examined in this study, even when prepared in PBST, vortexed and passed repeatedly through a fine gauge needle still had many large clumps,
so much so that counting of individual cells in stained smears was impossible. If other bovine strains behave similarly to 316V, then the reported numbers of organisms used for experimental infections with bovine strains and enumerated by plate culture may be inaccurate. Each clump would be counted as a single unit and significant underestimation would be likely.

2.2. The effect of 0.1% Tween-80 in serial dilutions used to provide MPN estimates for M. a. paratuberculosis (ovine strain)

Introduction and aims

Previous work with suspensions of cultured M. a. paratuberculosis (ovine strain) revealed a trend for MPN estimations to be less than the total numbers observed in direct counts. The difference between the two enumeration techniques varied from zero up to 1.9 logs for suspensions prepared in the same way. The reduction in MPN estimates may be due to reduced viability of the organisms (ie the MPN is giving a true indication of viable numbers), but could also be due to differences in the degree of clumping which occurs. Valid MPN estimations require that organisms are present as single units. If clumping is present, the MPN will underestimate the true count. While the ovine strains of M. a. paratuberculosis emulsify easily in PBS and minimal clumping is apparent when direct counts are performed on relatively concentrated suspensions, the possibility remains that significant clumping may occur in the serial dilutions used to detect the end point. Moreover, when MPN estimations are used on clinical samples, the association of M. a. paratuberculosis with cells or debris may exacerbate the problem.

In this experiment the MPN estimations and direct counts were compared for a suspension of cultured M. a. paratuberculosis before and after treatments to reduce clumping, and MPN estimations compared for faecal cultures where serial dilutions were prepared with or without Tween-80 to reduce clumping.

Methods

Suspensions from cultured M. a. paratuberculosis:

Undiluted suspension (S) was prepared from a modified 7H10 slope (pass 5) as previously described, thoroughly mixed by vortexing and divided into 2 aliquots. Serial 10-fold dilutions of one aliquot were made in PBS, vortexing for 30s between each dilution step (designated S-PBS). The second aliquot was diluted 10-fold in PBS with 0.1% v/v Tween-80 (PBST), vortexed for 30s, then passed 10 times through a 26 g needle and filtered through a 8μm filter to minimise any clumping of organisms. Subsequent dilutions in PBST were made from this preparation, again vortexing between each step (designated S-PBST; this was the same
suspension as 6b in Section 1). Direct counts, ZN smears and counts, and MPN estimations (10\(^2\) to 10\(^8\) dilution) were performed.

**Faecal samples:**

Two different samples (designated N and J) were processed routinely for Bactec culture. These were separate samples of heavily infected ovine faeces used in a pasture contamination experiment. Serial 10-fold dilutions from material resuspended in VAN were made in PBS or PBST, and MPN estimates (10\(^3\) to 10\(^9\) dilutions) performed.

**Statistical analysis:**

The MPN estimates obtained from serial dilutions in either PBS or PBST were compared using Student’s paired T test.

**Results**

**Direct counts and ZN stained smears:**

The 10\(^2\) dilution was used for direct counts. For S-PBS this dilution contained 7.89 organisms and for S-PBST 7.63, expressed as log\(_{10}\) number of organisms per mL. Thus the direct count for undiluted S-PBS was 9.89 organisms, and for the equivalent undiluted S-PBST was 9.63. There was little difference in subjective visual assessment of the degree of clumping in the counting chamber between the two suspensions, with the majority of bacterial cells present as apparently single cells, and only occasional clumps of two or three organisms. In the ZN smears clumping was more prominent. For suspension S-PBS the bacilli/“bacterial units” ratio was 1.6. For S-PBST this ratio was 1.1.

**MPN estimations:**

Results (as log\(_{10}\) numbers per mL of undiluted suspension) are given in Figure 2.2.1. For S-PBST growth was obtained in all Bactec vials at the 10\(^8\) dilution, so that the results recorded are minimum values. (The upper limit for S-PBST could be expected to be the direct count value of 9.63.) The mean of the MPN estimates obtained when serial dilutions were prepared in PBST was significantly greater than that when dilutions were prepared in PBS (P = 0.016).
Discussion

In both cultured suspensions and faeces the use of the extra treatments to reduce clumping increased MPN estimations. In the case of suspensions, although S-PBST had fewer total organisms than did S-PBS (the remainder presumably removed by filtration) when assessed by direct count, the MPN estimation for S-PBST was at least 1.3 logs higher than that for S-PBS. This difference was highly significant in that the 95% confidence intervals for the two MPN estimates did not overlap. For both faecal samples a similar result was obtained, with the MPN when serial dilutions were prepared in PBST almost a log higher than, and outside the 95% confidence intervals of the MPN estimations when dilutions were prepared in PBS. This effect was probably due to the reduction of clumping in the MPN estimations for the samples diluted in PBST. An alternative explanation could be that Tween-80 has a direct positive effect on the viability of \textit{M. a. paratuberculosis}. Tween-80 has been shown to have a stimulatory effect on the growth of \textit{M. a. paratuberculosis} in radiometric media at the levels used in the media in this study (0.002%), but whether this stimulatory effect on growth extends to an actual effect on viability of organisms at the critical dilutions is not clear.
If the effects observed are due to reductions in clumping it is apparent that subjective assessment of the degree of clumping from the wet preparations used in the direct counts is misleading since there appeared to be little difference between suspensions prepared in PBS or PBST. This could be the result of using Tween-20 to assist in the counting of routinely prepared PBS suspensions, although previous work had also shown little subjective difference between samples with and without Tween-20 (Section 1 of this chapter). The results of the ZN smears were more useful in this regard. In this experiment the numbers of individual bacilli were almost the same as numbers of “bacterial units” in smears from suspension treated to remove clumps, whereas in smears from suspension prepared in PBS alone there were 60% more individual bacilli than “bacterial units”. Unfortunately stained smears are of no use to assess the degree of clumping in the critical dilutions at the end-point in MPN estimations, where approximately one viable organism is contained in 100μL of suspension.

Regardless of the exact mechanism for the effect of Tween-80 on increasing the MPN estimates it is clear that the use of PBST rather than PBS in the serial dilutions used in MPN estimations provides a higher and probably more accurate estimate of the numbers of viable organisms present in the original sample. All subsequent MPN estimations for M. a. paratuberculosis in our laboratory have been done using PBST.

2.3. Use of cumulative growth indices in Bactec vials for the enumeration of ovine strains of M. a. paratuberculosis

Introduction and aims

Lambrecht et al described a mathematical model for estimating from CGI and incubation time the numbers of viable M. a. paratuberculosis of a laboratory-adapted bovine strain inoculated into a single Bactec vial. The model requires stringent conditions (a defined strain at a particular stage of growth in defined media and present as mainly single cells), and regression coefficients need to be established for each application by generating growth curves for the whole of the growth cycle. However, once these conditions are met it can accurately predict the numbers of organisms using CGI from a single Bactec vial, and has particular application in areas such as antimicrobial sensitivity testing. The method has also been used to quantify M. a. paratuberculosis in bovine faeces, and in cultured macrophages in vitro. For the purposes of pathogenesis studies in sheep, or quantification of M. a. paratuberculosis in sheep tissues, faeces or the environment a simpler technique applicable across a range of strains and sample types is needed. For these purposes the precision of the Lambrecht model is not necessary, and in many cases the conditions for valid application of the model cannot be met. A 95% confidence
interval of about one log would be acceptable – this is about the same as is provided by an MPN estimate which is currently the only technique available for determination of viable numbers of ovine strains of *M. a. paratuberculosis*. Detection times for *M. a. paratuberculosis* in radiometric culture (simply the number of days for a positive GI to be observed) for bovine faecal specimens have been reported to correlate with severity of disease, and by inference with numbers of organisms. Greater numbers of organisms in the sample were associated with shorter detection times. A similar relationship has been observed for Bactec cultures from ovine faeces, and in the sequential dilutions of *M. a. paratuberculosis* (ovine strains) used to provide MPN estimations described in Section 1. In this case the number of weeks taken for the weekly GI measurements to reach 999 was inversely correlated with the numbers of organisms inoculated. These observations suggest that direct radiometric measurements might be used to quantify ovine strains of *M. a. paratuberculosis* from a variety of sources.

Direct radiometric measurement would offer several potential advantages over MPN estimations. These include cost (one or several Bactec vials compared to 20 or more for MPN estimates), time (results in as little as one or two weeks when many organisms are present as compared to 12 weeks for MPN estimates), and being less affected by clumping (end-points in MPN trials would be reached at lower dilutions if significant clumping is present).

In this study GI over time were recorded for the whole of the growth curve in Bactec cultures of serial dilutions of suspensions of cultured ovine strains of *M. a. paratuberculosis*. We sought to establish a cut-off value for CGI which would reliably discriminate between inocula of different sizes, and in particular whether CGI of 1000 was useful. Such a cut-off would also facilitate the use of routine weekly GI readings to roughly estimate numbers of *M. a. paratuberculosis* because the Bactec 460 machine reads only up to a GI of 999. Decontaminated suspensions obtained from sheep tissues were then similarly examined to assess any effect of decontamination procedures and variation across a number of different isolates. This was expanded to include suspensions obtained from faeces and contaminated pasture/soils by re-examining MPN data from other investigations. The effect of source of sample (culture, tissue, faeces or soil), and the effect of Tween-80 in the dilutions used to produce MPN estimations, on the relationship between CGI and inoculum size was examined.
Methods

CULTURED M. A. PARATUBERCULOSIS AND SUSPENSIONS OBTAINED FROM INFECTED OVINE TISSUES:

Data from the MPN estimations of suspensions of cultured *M. a. paratuberculosis* (6a (bovine 361V), 6b and 6d) and suspensions processed directly from tissues (7a, 7b, 7c, 7e, 7f, 7g, 7h) as described in Section 1 were used in this study. MPN estimations (log$_{10}$) were 9.73 (6a), > 9.21 (6b), 7.96 (6d), 8.04 (7a), 9.23 (7b), 4.23 (7c), 3.85 (7e), 0.7 (7f), >5.21 (7g) and 2.11 (7h). The number of viable organisms inoculated into Bactec vials at each dilution used in each MPN trial was calculated from the MPN estimate, the dilution factor and the inoculum volume. In these MPN trials GI were measured as often as necessary to prevent readings going off-scale. During the period of maximum growth (lasting several weeks) this required readings every 1-3 days. CGI was calculated and mean CGI for the five replicate vials at each dilution were plotted against days of incubation across the whole of the growth cycle (initial lag phase, growth phase and static phase). For each trial, simple linear regressions of inoculum size on the number of days for mean CGI to reach 1000 (CGI1000), with 95% confidence intervals and 95% prediction bands, were performed using Minitab statistical software. For the tissue cultures, in addition to the routine IS900 PCR and REA for confirmation of *M. a. paratuberculosis*, gram and ZN stained smears of the contents of selected Bactec vials were examined for the presence of contaminant micro-organisms. Also, PCR for IS1311 and REA were performed as previously described to confirm that all isolates were typical ovine strains.

Weekly GI data only were available from Trials 1-5 (section 1) and were treated in the same way as the data for MPN trials on faeces (below).

SUSPENSIONS OBTAINED FROM OVINE FAECES OR CONTAMINATED PASTURE/SOIL:

Data from MPN estimations (performed as part of other research projects) on faeces or faecally contaminated pasture/soils, and for which only weekly GI had been measured, were re-examined in this study. In each MPN trial the lowest dilution level (i.e. that with the highest inoculum size) was examined. Inoculum size was calculated as above and the CGI1000 was extrapolated from the weekly data as follows. For each of the replicate Bactec vials CGI for the week before the weekly GI exceeded 999 was fitted to a “typical” growth curve obtained from cultured ovine *M. a. paratuberculosis*. The number of days taken for the “typical” curve to reach a CGI of 1000 from the observed CGI level was then added to the time of the weekly
observation in question. The median CGI1000 from the replicate vials was the value used for analysis.

**Statistical methods:**

*Use of CGI1000 to estimate inoculum size:* Trial 6d was chosen for detailed examination of the validity of using CGI1000 as a cut-off value to discriminate among inoculum sizes, specifically to compare CGI1000 with CGI2000, CGI3000, up to CGI10000. Plots of log_{10} inoculum size against these criteria showed them to be predominantly linear for all trials examined, so the discrimination method chosen was linear discrimination among the log_{10} dilution factors (-1 to -6). The time trend for each replicate was modelled using a cubic smoothing spline. Bivariate linear regressions on the log_{10} dilution factors were performed for the nine pairs of days corresponding to CGI1000 and one each of CGI2000 to CGI10000. Step-down F-ratios for the members of each pair were obtained to assess the significance of removing each member from the pair. If the removal of one critical value term was significant but removal of the other was not, the latter was assumed not to contribute significantly to discrimination among the dilutions in the presence of the former.

*Relationship of CGI1000 to inoculum size for samples from different sources prepared with or without Tween-80:* Sources of samples included cultures, tissues, faeces and faecally contaminated pasture/soil (soils). The log_{10} inoculum size and the CGI1000 from 5 replicate vials were considered for each sample. When one of the 5 vials had no growth the median of the 5 CGI1000 values (3rd ranked value) was recorded for the sample and the remaining CGI1000 values were ignored. When two or more vials had no growth the sample was excluded from the regression modelling. The resulting sample sizes were 9, 6, 8 and 42 for cultures, tissues, faeces and soils respectively. The number of samples prepared with/without Tween-80 were 10/13 for cultures, tissues and faeces and 24/18 for soils. The CGI1000 values were considered to be dependent on the log_{10} inoculum size of the sample and so were the dependent variable in regression modelling. Linear mixed models were used to assess (a) linear and curvature effects of log_{10} inoculum size, with curvatures estimated as random effects using cubic smoothing splines; (b) effects of source of sample and their interactions with the linear effects and curvature effects of log_{10} inoculum size; (c) the effect of Tween-80 and its interaction with the linear effects and curvature effects of log_{10} inoculum size; (d) an interaction between the source effects and Tween-80 and its further interaction with the linear effect of log_{10} inoculum size; and (e) the random effects of vials within samples for each source and the random effects of samples within source for each source. The full linear model comprising the effects and interactions (a) to (e) was reduced to a final model by successive elimination of non-significant
terms, using a significance level of 5% (P<0.05) unless stated otherwise. All analyses were performed using ASReml$^{10}$. Equations for predicting log$_{10}$ inoculum size from CGI1000, with 95% confidence limits for predicted means and 95% prediction limits for single vials, were determined by inverting the fitted relations and their confidence and prediction limits.

Results

No contaminants were detected in the tissue cultures and all had REA profiles after IS1311 PCR which were typical of ovine M. a. paratuberculosis. All trials generated similar shaped growth curves. As an example, the results for Trial 6d are shown in Figures 2.3.1a and b.

Figure 2.3.1a: Trial 6d: Cumulative growth index for each Bactec vial

![Cumulative growth index for each Bactec vial](image-url)
USE OF CGI1000 TO ESTIMATE INOCULUM SIZE:

For Trial 6d the univariate regressions of CGI1000 to CGI10000 on dilution factors –1 to –6 were all strongly linear and negative with very high $r^2$ values (98.8, 98.6, 97.7, 97.4, 97.1, 96.4, 94.2, 93.3, 91.0 and 85.1 for CGI1000 to CGI10000 respectively). From the bivariate regressions, the step-down F-ratios for CGI1000 were significant for every pair ($P<0.05$ with CGI2000, $P<0.001$ with CGI3000 to CGI10000), but the step-down F-ratios for the other members of the pairs were all non-significant ($P>0.2$). This indicates that CGI2000 to CGI10000 do not provide any additional information to discriminate among the inoculum sizes above that provided by CGI1000 alone. Figures 2.3.2a and b (simply enlargements of the relevant parts of Figures 2.3.1) show the early part of the growth curves for Trial 6d, demonstrating visually the good discrimination between inoculum sizes at CGI1000.
Inoculum sizes (log10) range from 6 for the $10^{-1}$ dilution to 0 for the $10^{-7}$ dilution.

Figure 2.3.2a: Trial 6d: Cumulative growth index for each Bactec vial

Figure 2.3.2b. Trial 6d: Mean cumulative growth index (means of five replicate samples)

Linear regression of log10 inoculum size on CGI1000 for a specific isolate/MPN trial:

Again Trial 6d is used as an example. From Figure 2.3.2b the mean CGI1000 are obtained by taking the x value for each curve when y = 1000. Figures 2.3.3a and b illustrate the resulting simple linear regression of inoculum size on mean CGI1000 for Trial 6d. In Figure 2.3.3a the
95% confidence interval and 95% prediction bands are shown for estimation of inoculum size from the mean CGI1000 of 5 replicate vials. The 95% prediction bands are about 0.6 of a log above and below the predicted inoculum size for an observed CGI1000. In Figure 2.3.3b the x error bars represent the range of CGI1000 across the five replicate vials. It is apparent that for inoculum sizes above \(10^2\) organisms, the ranges of estimated inoculum sizes predicted from regression on CGI1000 in a single Bactec vial are within the 95% confidence limits of the MPN-based estimates of inoculum size. As the inoculum size decreases the ranges increase. For inula of \(10^2\) or \(10^1\) organisms the ranges are of about the same magnitude as the confidence limits of the MPN estimation. As would be expected, with a calculated inoculum size of just one organism, ranges are larger (and some vials did not have any growth at all).

Much of this variation in CGI1000 at small inoculum sizes represents the variation in inoculum size due to chance rather than variation in CGI1000 across vials with the same size inoculum. At lower dilutions (ie larger numbers of organisms) such effects are negligible, and the CGI1000 from a single vial could reasonably be used to obtain an estimation of inoculum size.

**Figure 2.3.3a. Trial 6d: Inoculum size vs CGI1000**

mean of 5 replicate vials, with 95% confidence intervals (....) and 95% prediction bands (----)

![Graph showing the relationship between log10 inoculum size and days post inoculation for CGI to reach 1000](image)
The regressions with 95% prediction bands for Trials 6a and 6b are shown in Figure 2.3.4. These relationships were used in the temperature-survival study described in Section 4 of this chapter. The 95% prediction bands are about 0.9 and 0.2 of a log above and below the predicted inoculum size for an observed CGI1000 for Trials 6a and 6b respectively.
The relationships of calculated inoculum size with CGI1000 for *M. a. paratuberculosis* isolated directly from ovine tissues (7a,7b,7c,7e,7f,7g,7h), with fitted line plots for trials with 3 or more data points, are shown together with those for cultures (6a, 6b, 6d) in Figure 2.3.5. For each tissue isolate, results were similar to those obtained for cultured *M. a. paratuberculosis*, displaying a predominantly linear relationship between inoculum size and cgi1000 with high $r^2$ values (98.3 (6a), 99.8 (6b), 99.3 (6d), 99.8 (7a), 99.7 (7b), 92.5 (7c), 97.1 (7e), 98.5 (7g)). It is notable that the bovine isolate (Trial 6a) was quite distinct from most ovine isolates, as was ovine isolate 7c. These two isolates took longer to reach a CGI of 1000 than the other ovine isolates.
PREDICTION OF $\log_{10}$ INOCULUM SIZE FROM CGI1000 FROM UNCHARACTERISED SAMPLES:

Weekly GI were available from MPN trials on *M. a. paratuberculosis* isolated from pooled faeces from known infected sheep (6 performed with dilutions in PBS, and 2 in PBST), from faecally contaminated pasture/soil (20 with dilutions in PBS and 30 in PBST), and from other cultured suspensions (Section 1; 5 performed with dilutions in PBS). The CGI1000 and the inoculum size for each separate MPN trial are each plotted as single points in Figure 2.3.6, along with data for the highest calculated inoculum size in each MPN from Trials 6 (ovine strains only) and 7. Where calculated inoculum size ($\log_{10}$) was less than 0 (such MPN trials all had some vials with no growth at the lowest dilution), an inoculum size of one organism (0 $\log_{10}$) was assumed, because the median CGI1000 was from a vial with growth (and obviously had an inoculum size of at least one organism). The approximately linear relationship of CGI1000 with inoculum size across the wide range of sample sources is apparent.
Figure 2.3.6. Relationship of CGI1000 to inoculum size for different isolates of *M. a. paratuberculosis* (ovine strains)
(Each point represents a separate MPN trial)

Detailed statistical analysis: There was a strong negative linear relation between CGI1000 and log_{10} inoculum size (P<0.001) with no significant curvature (P>0.50), in which the slope of the relation did not differ significantly between sources (P>0.50) or due to an effect of Tween-80 (P>0.50). The effect of Tween-80 on the intercept of the relation was positive and significant (P<0.001). Among the sources, the intercept for the soil samples was higher (P<0.10) than that for the non-soil samples (cultures, tissues and faeces). There were marked differences in the random effects of vials and samples on CGI1000 between the soil and non-soil sources (P<0.001). The variance component for vials within samples for the non-soil sources was 1.71, and that for the soil source was 17.74. The higher variance among soil samples is likely to be due in part to the lower inoculum sizes for the soil samples, for which the variation of CGI1000 across replicate vials would be expected to be large, as illustrated in Figure 2.3.3b, and also the use of dilution series from 5 sub-samples in the MPN estimations from soils. The variance component for samples for the non-soil sources was 13.23, and that for the soil source was 8.01, the difference mainly a result of the greater numbers of soil samples available for analysis. The total variances for the soil and non-soil data were 25.75 and 14.94, respectively. In the final model relating CGI1000 to log_{10} inoculum size the average intercept was 46.82±1.20, the overall slope was -5.31±0.40, the difference between Tween-80 and non-Tween-80 samples.
was 3.24±0.92 and the difference between soil and non-soil sources was 2.69±1.54. The
intercepts for the four Tween/non-Tween x soil/non-soil lines (all with the same slope) were:
Tween/soil  49.79±0.85, Tween/non-soil 47.10±1.97, non-Tween/soil 46.55±0.91 and non-
Tween/non-soil  43.85±1.88. Figure 2.3.7 illustrates the above model with lower and upper
95% confidence limits and lower and upper 95% prediction limits from single Bactec vials for
samples from non-soil and soil sources.

**Figure 2.3.7. Relationship of inoculum size to
time for CGI to reach 1000**

![Graph showing relationship between inoculum size and days for CGI to reach 1000](image)

Inoculum size was standardised against MPN estimates with dilutions in PBST.

"Non-soils" includes samples from cultured organisms and from decontaminated suspensions
from faeces or tissues

**Discussion**

In this study the CGI1000 was shown to be more useful than data from the whole of the
growth curve in establishing a relationship with inoculum size. This means that Bactec cultures
of *M. a. paratuberculosis* (ovine strains) can be read weekly until evidence of growth is detected,
then every 2 or 3 days for about a further week until CGI exceeds 1000, without readings going
off-scale, making the procedure practical for routine laboratory use. At this CGI value it was also possible to extrapolate an approximate CGI1000 value from weekly GI readings, allowing retrospective estimation of inoculum sizes from routine cultures.

For each isolate of ovine \textit{M. a. paratuberculosis} which was studied in detail, a predominantly linear relationship was observed between inoculum size and mean CGI1000 of 5 replicate Bactec vials, with 95% prediction bands of +/- one log or less. Thus, once an isolate has been characterised in an MPN estimation, and a regression of inoculum size on CGI1000 established, the measurement of mean CGI1000 from replicate Bactec vials becomes an accurate, economical and practical alternative to MPN estimation for on-going studies involving that particular isolate. Also, because the range of CGI1000 across replicate Bactec vials is small for inoculum sizes above $10^2$ organisms, measurement of CGI1000 from even a single vial can provide a good indication of inoculum size.

An approximately linear relationship was also demonstrated across isolates from a variety of sources with 95% prediction bands about 1.5 to 2 logs on either side. An effect of the use of Tween-80 in increasing the observed CGI for a given inoculum size was seen, consistent with the results of the earlier study (Section 2 of this chapter). The MPN estimations when dilutions are prepared in PBST are a better estimate of viable numbers, and so the final prediction equations were expressed in these terms. The detailed linear regression modelling allowed all the available data (from PBS as well as PBST trials) to be used in the development of this predictive relationship. There were no significant differences between non-soil sources of samples (cultured \textit{M. a. paratuberculosis}, or decontaminated suspensions obtained from tissues or faeces), so the same prediction relationship could be used for such samples. The soil samples were different, however, taking longer to reach a CGI of 1000 than other samples with similar inoculum sizes, and so requiring a separate predictive relationship. The many other sources of potential variation (such as genetic differences between isolates, differences due to stage of disease (see below), or those due to sample storage) have not been examined. However, with these caveats, the established relationship has been useful in our laboratory to provide estimates of the number of organisms present in clinical samples using data extrapolated from routine Bactec cultures.

A subjective examination of Figure 2.3.5 suggests that the laboratory adapted bovine strain of \textit{M. a. paratuberculosis} (316V) behaved quite differently in culture compared to the ovine strains.
The ovine isolate from Trial 7c was also distinct from the other ovine isolates in this study. It is interesting that 7c was the only isolate examined from a paucibacillary case of OJD. A second trial from another paucibacillary case was set up (Trial 7d) but *M. a. paratuberculosis* failed to grow in any culture. It is possible that many *M. a. paratuberculosis* organisms in paucibacillary cases exist in an “inhibited” form, and may take longer to commence to grow in Bactec culture than cultured organisms or those from multibacillary cases. Some authors have suggested that they may exist as cell-wall deficient forms,144 thus accounting also for the failure of ZN stains to detect them in many cases. Further work on isolates from paucibacillary cases is needed to confirm this isolated observation. The results for *M. a. paratuberculosis* isolated from soil samples may reflect a similar phenomenon. These organisms also took longer to grow in Bactec culture than similar numbers of *M. a. paratuberculosis* from tissues, faeces or cultures. Does the exposure to adverse environmental influences have an inhibitory effect on the initial ability of the organism to multiply in culture?

A potential hazard in the use of CGI1000 in the enumeration of *M. a. paratuberculosis* from clinical samples is contamination of the Bactec cultures. This can be sufficiently severe to prevent identification of *M. a. paratuberculosis* even if present. Rates for such contamination amongst routine cultures in the EMAI laboratory have been negligible for tissue cultures and less than 5% for faecal cultures (Whittington unpublished). In some experimental situations contamination rates can be very low (eg. none of 600 tissue cultures were contaminated in the pen trials described in Chapter 3) or much higher (in some groups of sheep examined in field trials described in Chapter 4 contamination rates in excess of 20% were found). Published contamination rates for faecal cultures range from about 4 to 11%71 153 158 Such contamination, while a problem for isolation, is easily recognised and unlikely to confuse enumeration attempts. However, “co-contamination”, where some cultures have coincident growth of other organisms which contributes to the observed GI but does not prevent the confirmation of *M. a. paratuberculosis*, may also occur. Thus, to use CGI measurements to estimate inoculum size it is necessary to confirm, not only with PCR and REA that *M. a. paratuberculosis* is present in the Bactec vials, but also that significant growth of other organisms is not present. Possible co-contamination needs to be considered when data from routine Bactec cultures (which have rarely been examined for co-contamination) are retrospectively assessed. Co-contamination is not a problem in MPN estimations, where it is only necessary to confirm that *M. a. paratuberculosis* is present in all vials with positive GI at the critical dilutions.
2.4. The effect of storage 4 °C, -20 °C or -80 °C on the survival of cultured M. a. paratuberculosis in PBST suspensions

Introduction and aims

In experimental infections with M. a. paratuberculosis the dose of viable organisms is usually determined retrospectively (if at all) by some form of quantitative culture. This was done using MPN estimations for the PBS suspensions of M. a. paratuberculosis which were used to infect sheep experimentally as described in Chapter 3. In this case the actual doses of viable organisms administered were found to be about a log below those originally planned. Enumeration of numbers of viable organisms in suspensions and subsequent storage of those suspensions without loss (or at least with defined loss) of viability would greatly facilitate the planning of experimental infections and the comparison of effects between different investigations without the confounding effects of dosage variation. Reliable storage would also facilitate experimental manipulations of ovine strains of M. a. paratuberculosis in the laboratory. Bovine strains of M. a. paratuberculosis in 7H9 broth can be stored, apparently without loss of numbers, at -70 °C, but there is no published work on the survival of ovine strains. The MPN investigations used to quantify the experimental inocula described in Chapter 3 indicated some loss in numbers of viable organisms in PBS suspensions over several weeks storage at 4 °C (Appendix 3a). Earlier work (Whittington, unpublished) has shown good survival of the ovine strains of M. a. paratuberculosis for at least 2 years in Bactec medium at -20 °C and -80 °C. Observations of the numbers of weeks taken for the weekly GI to exceed 999, and based on the findings in Section 3 of this chapter, suggested that numbers had remained stable during such storage. The high percentage of egg yolk in the medium may have had a cryoprotective effect.

The aim of this study was to assess the survival of M. a. paratuberculosis in PBST suspensions over time at 4°C, and at -20 °C and -80 °C with and without the cryoprotectant glycerol. In addition, filtered and unfiltered suspensions were examined to assess whether the small clumps of organisms in unfiltered material affected survival at these temperatures.

Methods

Suspended M. a. paratuberculosis:

Suspensions were prepared from selected undiluted suspensions as detailed in Section 1, and dilutions prepared in PBST or PBST with 20% v/v glycerol. These were 6a (bovine, 316V, 1:100 dilution), 6b (5th passage ex ovine faeces, 1:100 dilution), 6d (2nd passage ex ilium of
experimentally infected sheep, 1:1000 dilution). Each suspension was assessed in filtered or unfiltered form (i.e., a total of six different suspensions).

**EXPERIMENTAL DESIGN:**

Ten 0.5 mL aliquots in 1.0 mL cryotubes were prepared for each suspension, six without and four with glycerol. There were five separate storage treatments with two aliquots (A and B) of each suspension prepared for each treatment:

1. 4 °C
2. -20 °C
3. -80 °C
4. -20 °C with 20% v/v glycerol
5. -80 °C with 20% v/v glycerol

On the day of preparation of the suspensions, each suspension was assessed to provide baseline results. Subsequently, each suspension was assessed after storage for 3, 6, and 13 months. (Further assessments at 24 and 36 months are planned.) Aliquots A were sampled at 3 and 13 months and aliquots B at 6 months. Thus, suspensions assessed at 3 and 6 months had undergone one freeze/thaw cycle, while those assessed at 13 months had undergone 2 freeze/thaw cycles.

**ASSESSMENT OF VIABILITY AND NUMBERS OF M. A. PARATUBERCULOSIS:**

At each time period, a 100μL aliquot from each suspension was inoculated into a Bactec vial, incubated, and read as previously described. Growth indices were read weekly until growth was observed, then three times weekly until the CGI exceeded 1000. Vials without observed GI were read for at least 12 weeks to confirm negative results. The approximate number of viable organisms was determined
from the CGI1000 using the regressions already established for those particular isolates, as described in Section 3.

Results

Figure 2.4.1 shows the changes in numbers of viable organisms over the first 13 months for each of the six suspensions. Estimated initial log_{10} numbers in the suspensions before storage ranged from 8.1 in 6a(unfiltered) to 2.9 in 6d(filtered). The 6 suspensions all showed similar trends, regardless of their species of origin, initial numbers of organisms or whether filtered or unfiltered, so data for the six suspensions were pooled to yield the composite graph shown in Figure 2.4.2.
Figure 2.4.1. Survival in PBST +/- glycerol of *M. a. paratuberculosis* at different temperatures

**Trial 6a. Bovine laboratory adapted strain 316V**

Unfiltered suspensions

Filtering suspensions

**Trial 6b. Ovine strain, originally isolated from faeces**

Unfiltered suspensions

Filtering suspensions

**Trial 6d. Ovine strain, originally isolated from tissues**

Unfiltered suspensions

Filtering suspensions
Figure 2.4.2. Survival in PBST +/- glycerol of *M. a. paratuberculosis* at different temperatures

(Pooled results from all six suspensions examined)

There was minimal loss of viability (less than one log) for suspensions stored at -80 °C, or for suspensions with added glycerol frozen at either temperature. Suspensions stored at 4 °C showed a steady loss of about 3 logs over the first 6 months. By 12 months only the unfiltered suspension 6b still had viable organisms, having lost 5.5 logs over that time. The 2 bovine suspensions had lost at least 8.1 and 7.6 logs over that same period. At -20 °C, loss in suspensions without added glycerol was intermediate to the losses at –80 °C and 4 °C.

**Discussion**

Storage at -80 °C with or without added glycerol for up to 13 months resulted in minimal reduction in numbers of viable *M. a. paratuberculosis* stored in PBST. The rapid freezing which presumably occurs with small sized samples exposed to -80 °C would not appear to be deleterious to the organism. At -20 °C there was reduction of about one to two logs over the first 3 months, with a reduced rate of loss thereafter. Addition of glycerol was cryoprotective as expected, and the glycerol-treated samples stored at -20 °C showed little loss of numbers over the 13 months. Together these findings suggest that the initial freezing at -20 °C may be more significant for loss of numbers than the actual length of storage at -20°C.

In this study there was no evidence that clumping of *M. a. paratuberculosis* was protective during storage since filtered and unfiltered suspensions of the same isolates had about the same rates of loss.
The finding that storage at -80 °C preserves viable numbers of organisms of *M. a. paratuberculosis* (ovine strains) will facilitate accurate and repeatable doses of infectious organisms in future experimental infections.

2.5. The effect of decontamination protocols on the numbers of *M. a. paratuberculosis* of ovine origin isolated from tissues and faeces

*Introduction and aims*

The possible effects on numbers of viable organisms of decontamination protocols used in the culture of *M. a. paratuberculosis* from clinical samples were discussed in the general introduction to this chapter. Such effects may reduce numbers by one or more logs, and may result in false negative cultures. There are no published reports for the effects of decontamination procedures on ovine strains of *M. a. paratuberculosis* which have only recently been reliably cultured in liquid media. In this study, the effect on numbers of *M. a. paratuberculosis* (ovine strain) of exposure to decontamination procedures for tissues and faeces spiked at selected points during preparation was assessed by measurement of CGI1000 in Bactec cultures (Section 3). The decontamination procedures assessed were those in routine use at the EMAI laboratory, and in addition a variation to the tissue protocol using a final centrifugation step. The performance (sensitivity and contamination levels) of the routine and centrifuged tissue decontamination protocols were also compared across a range of naturally infected tissue samples.

*Methods*

**Experiment 1. Spiked tissue and faecal samples:**

*Preparation of tissue and faecal samples for the spiking experiment:*

Terminal ileum and faeces were collected at necropsy from several sheep from a flock monitored negative for ovine Johne's disease and stored at -80 °C until processed. The samples were thawed, pooled to produce one pool of tissues and one of faeces, homogenised with a small amount of saline in a blender, dispensed into 2mL aliquots and stored at -80 °C until required. Prior to the main experiment several aliquots of tissue and faeces were thawed, processed by the routine decontamination procedures and cultured in Bactec vials to confirm negative status for *M. a. paratuberculosis*, and also to assess potential contamination of cultures.

*Preparation of stock suspension of *M. a. paratuberculosis*:*

*M. a. paratuberculosis* from previous experiments (Section 1, Trial 6b, 5th passage of material originally isolated from faeces of infected sheep) was stored at −80 °C in modified Bactec medium. This was thawed, reinoculated into modified Bactec medium, and 7H10 slopes were
inoculated from the fresh Bactec vial when the weekly GI reached 999. Colonies were harvested in PBST as previously described from four 6 week old slopes. The resulting suspension was expected to contain about $10^{10}$ organisms per mL (based on experience of the preparation of similar suspensions in Section 1). This was diluted tenfold in PBST, vortexed for 1 min, passed 10x through a 26g needle, then filtered through an 8 μm filter. The resulting suspension was further diluted 1:10 in PBST, dispensed into 1 mL aliquots (expected to contain about $10^8$ organisms per mL) and stored at −80 °C. The stored material was termed stock suspension. The total number of organisms was determined by direct counts in a Thoma chamber as previously described. A single aliquot was thawed and triplicate Bactec vials inoculated with 100μL of the stock suspension to estimate viable numbers as described below. In addition triplicate Bactec vials were inoculated with each of the dilutions $10^{-4}$ to $10^{-8}$ of the stock suspension to obtain an MPN estimate, in this case using the number of *M. a. paratuberculosis* positive Bactec vials from the 3 vials inoculated from each of three serial 10-fold dilutions.\(^{15}\)

**Experimental design:**

There were eight treatment groups, each inoculated with *M. a. paratuberculosis* at a different stage of the decontamination procedure as indicated below. For each treatment group four dilutions of stock suspension were used to span the range of numbers of organisms likely to be encountered in clinical samples, and triplicate Bactec vials were inoculated for each dilution of each treatment. Aliquots of the stock suspension were thawed at 4 °C within one hour of use and dilutions made in PBST. All of the samples were processed so that the final inoculation into Bactec vials was done at the same time. All faecal cultures (groups FE, FC, FB and FA) were prepared prior to inoculation of Bactec vials exactly as described in the general methods. After the final resuspension after 72 hours in 1mL VAN, three 100μL aliquots were removed from each culture preparation for inoculation of three replicate Bactec vials. Tissue cultures were prepared prior to inoculation of Bactec vials by either the routine method (TA) or centrifuged method (TE, TB) as described in the general methods. In the routine method three separate 100μL aliquots were carefully removed from near the bottom of each tube after 72 hours sedimentation in 0.75% HPC. In the centrifuged method the three 100μL aliquots were removed from the material resuspended in 1 mL of HPC.

**C:** (Control) Bactec vials were inoculated directly with bacterial suspensions.

**FE:** (Faeces, end) Faecal preparations spiked at the end of the decontamination procedure. Bacterial suspensions were added to the 1 mL of resuspended VAN immediately before
removal of aliquots for inoculation of Bactec vials. Thus the *M. a. paratuberculosis* organisms were exposed to VAN for a brief period, plus a small carryover of VAN to the inoculated Bactec vials.

**FC:** (Faeces C) Faecal preparations spiked immediately after material was first resuspended in VAN after 24 hours in HPC/BHI. Organisms in this treatment were exposed to VAN for 72 hours, plus carryover to the Bactec vials.

**FB:** (Faeces B) Faecal preparations spiked immediately after the 5ml saline aliquot was added to the HPC/BHI. Organisms were exposed to HPC/BHI for 24 hours, a centrifugation step, then VAN for 72 hours, plus carryover to the Bactec vials.

**FA:** (Faeces A) Faeces spiked 30 min before the start of the decontamination procedure and processed routinely as described in the general methods. Organisms undergo a sedimentation and sub-sampling effect, then as for FB.

**TE:** (Tissues end) Tissue preparations spiked at the end of the decontamination procedure. Bacterial suspensions were added to material resuspended in 1mL HPC immediately before inoculation of the Bactec vials. Organisms exposed briefly to HPC, plus carryover to Bactec vials.

**TB:** (Tissues B) Tissues spiked 30 min before the start of the decontamination procedure and processed by the centrifugation method. Organisms exposed to HPC for 72 hours, a centrifugation step, plus carryover to Bactec vials.

**TA:** (Tissues A) Tissues spiked 30 min before the start of the decontamination procedure and processed routinely. Organisms exposed to HPC for 72 hours, sedimentation and a sub-sampling effect, plus carryover to Bactec vials.

For group C 100μL of the 10⁻¹, 10⁻³, 10⁻⁵ and 10⁻⁷ dilutions of the stock suspension (expected to contain about 10⁶, 10⁴, 10² and 10⁰ organisms respectively) were used. For all groups F and T 100μL of the undiluted, 10⁻², 10⁻⁴ and 10⁻⁶ dilutions of the stock suspension (expected to contain about 10⁷, 10⁵, 10³ and 10¹ organisms respectively) were used. The use in groups F and T of a tenfold lower dilution (ie ten times more organisms) than group C reflects the inherent tenfold sub-sampling effect in the use of a 100μL aliquot from a 1mL final volume inoculated into the Bactec vials for the faecal cultures and for groups TE and TB from the tissue cultures. The sub-sampling factor in the routine tissue culture procedure where a 100μL aliquot is removed from “near the bottom” of about 30 mL of sedimented HPC suspension was unknown, but a tenfold factor was assumed to allow direct comparison with the other treatments.
ENUMERATION OF VIABLE M. A. PARATUBERCULOSIS FROM BACTEC VIALS:

Bactec vials were incubated at 37 °C for up to 12 weeks. Growth indices were measured weekly until growth was observed, then as often as necessary to prevent their going off scale, until the mean CGI of the three replicate vials exceeded 1000. The number of viable organisms was estimated from the mean CGI1000 using the regression established for Trial 6b in Section 3.

Experiment 2. Comparison of routine and centrifuged methods for naturally infected tissue samples:

Tissues obtained at necropsy (a subset of the Year 2, 5 month post-weaning samples from the field trials described in Chapter 4 of this thesis) were stored at –80 °C for up to 3 months until cultured. A total of 178 tissues were used in this study. Each tissue sample was homogenised in 2 mL of sterile saline and equal aliquots from the resulting homogenate were prepared in parallel by the routine and centrifuged tissue decontamination methods. A single Bactec vial was inoculated for each method, GI were read weekly for 12 weeks, and *M. a. paratuberculosis* identified in GI positive vials as described in the general methods. The number of days taken for the CGI to exceed 1000 in the cultures prepared by the centrifugation method was extrapolated from the weekly data as previously described (Section 3).

STATISTICAL ANALYSIS:

Sensitivity and contamination rates for the centrifugation and routine methods were compared using McNemar’s Chi-square test for paired observations. Students T test was used to compare the mean of CGI1000 of samples which were culture positive by both methods with that of samples positive by the centrifugation method only.

Results

Experiment 1:

Preliminary cultures of pooled faecal and tissue samples revealed no growth of *M. a. paratuberculosis* and contamination was not observed. The stock suspension of *M. a. paratuberculosis* contained 1.1 X 10^8 total organisms/mL as measured by direct counts and consisted mainly of single cells. The estimate for viable numbers of *M. a. paratuberculosis* using regression on CGI1000 was 7.8 X 10^7/mL, and using MPN estimation was 1.2 X 10^8/mL. The numbers of viable *M. a. paratuberculosis* remaining after the decontamination procedures are shown for faeces (Figure 2.5.1a) and tissues (Figure 2.5.1b). Similar results were obtained across the different levels of infection so the data for expected levels of 10^6, 10^4 and 10^2 inoculated organisms were pooled and the results are summarised in Table 1. The routine faecal
decontamination method reduced expected numbers of viable organisms by more than one log. The routine tissue decontamination method reduced numbers by about 2 logs, and the centrifuged method by about one log.
Figure 2.5.1a. Effect of decontamination procedures on numbers of viable \textit{M. a. paratuberculosis} in tissue samples

Figure 2.5.1b. Effect of decontamination procedures on numbers of viable \textit{M. a. paratuberculosis} in faecal samples

Values below the axis indicate no growth in any of the 3 replicate bactec vials at that inoculum level

* indicates no growth in some vials
Table 2.5.1. Effect of decontamination/concentration procedures on numbers of viable *M. a. paratuberculosis*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; reduction in numbers compared to controls (mean of samples with expected levels of 10&lt;sup&gt;6&lt;/sup&gt;, 10&lt;sup&gt;4&lt;/sup&gt; or 10&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Faecal culture procedures</strong></td>
<td></td>
</tr>
<tr>
<td>FE</td>
<td>Brief exposure to VAN</td>
</tr>
<tr>
<td>FC</td>
<td>72h exposure to VAN</td>
</tr>
<tr>
<td>FB</td>
<td>24h in HPC/BHI, then 72h exposure to VAN</td>
</tr>
<tr>
<td>FA</td>
<td>Routine EMAI faecal culture</td>
</tr>
<tr>
<td><strong>Tissue culture procedures</strong></td>
<td></td>
</tr>
<tr>
<td>TE</td>
<td>Brief exposure to HPC</td>
</tr>
<tr>
<td>TB</td>
<td>72h exposure to HPC, centrifugation/resuspension</td>
</tr>
<tr>
<td>TA</td>
<td>Routine EMAI tissue culture</td>
</tr>
</tbody>
</table>

Experiment 2:

Culture results for samples positive by either method are given in Table 2.5.2. Using the routine method 7/178 tissue samples were positive for *M. a. paratuberculosis*. With the centrifugation method 12/178 were positive, including all 7 which were positive by routine decontamination. Thus routine decontamination detected only 58% of tissue samples that were detected as positive by the centrifugation method. The difference was significant (Chi-square = 5.0, P < 0.05). Only 2/178 samples (1.1%) were contaminated (GI detected but negative for *M. a. paratuberculosis* by IS900 PCR) using the routine method, whereas 15/178 (8.4%) were contaminated using the centrifugation method. One sample was contaminated in both methods. The difference was very highly significant (Chi-square = 11.3, P < 0.001). The mean of CGI1000 (centrifugation method) for samples positive by both methods was 38.1 days, and that for samples positive only in the centrifugation method was 50.4 days. This difference approached significance in a one-tailed T test. (T = -1.81, P = 0.054).
Table 2.5.2. Comparison of routine and centrifugation protocols for recovery of *M. a. paratuberculosis* from ovine tissue samples (data for samples positive by either method)

<table>
<thead>
<tr>
<th>Culture result</th>
<th>Days for CGI to reach 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine decontamination</td>
<td>Centrifugation method</td>
</tr>
<tr>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>++</td>
<td>++</td>
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<tr>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Discussion

Until recently the only method available for quantification of ovine strains of *M. a. paratuberculosis* was end-point titration in liquid media. Such a technique was too expensive for the type of study described here. In Experiment 1 of this study, regression of inoculum size on CGI1000 for a particular isolate (6b, Figure 2.3.4) allowed quantification of the reduction in numbers of organisms in inocula after the various decontamination procedures from triplicate Bactec vials. MPN estimations would have required 6 to 10 times as many Bactec vials. In Experiment 2, CGI1000 from single Bactec vials was used directly to compare relative numbers of organisms and estimates for numbers could be obtained from the general predictive relationship depicted in Figure 2.3.7.

By comparing the results for samples spiked at the start of the decontamination procedures (Groups TA and FA) with the results for control cultures (Group C) the total effects of these procedures can be assessed. The routine procedures in use at EMAI for faecal and tissue culture for ovine strains of *M. a. paratuberculosis* resulted in about one and two log decreases in organism numbers respectively. Note that these figures already allow for a one log reduction due to the inherent sub-sampling effects in the procedures, so that the detection limit for routine faecal culture is about $10^2$ organisms in the original sample, and for routine tissue culture about $10^3$ organisms. Obviously when small numbers of organisms (less than $10^2$ for faeces or $10^3$ for tissues) are present in clinical samples false negative cultures will be frequent.
In the initial processing of faecal samples, there is a sedimentation step in a total volume of 12-14 mL of saline and faeces, with removal after 30 min of a 5mL aliquot from the surface layer. The effect of this step is revealed by comparison of the results for groups FA and FB. This step resulted in a minimal loss (0.3 log), about a two fold difference, in line with the simple sub-sample effect of taking 5 mL from a total volume of about 12 mL. In this experiment, faeces were spiked just 30 min before the start of decontamination. In real clinical samples *M. a. paratuberculosis* may be more closely associated with faecal solids which settle out, so this result may not reflect the situation for clinical samples (the loss of numbers from clinical samples may be greater). Even brief exposure to VAN followed by some carryover to the Bactec vials appeared to slightly reduce the numbers of organisms in the faecal cultures. The same effect was not seen for the 0.75% HPC used in the tissue decontamination protocol (compare Groups TE and FE with Group C). In the faeces protocol there was a further 1.2 log reduction in numbers following 72 hours in VAN (compare Group FC with FE), but there was no further reduction in numbers due to the 24h in HPC/BHI (compare Group FB with FC). In fact, there is some suggestion that this step may minimise the effects on *M. a. paratuberculosis* of the subsequent exposure to VAN. In the samples spiked with $10^2$ organisms, no growth was seen in any of the three Bactec vials following exposure to VAN alone for 72h (Group FC), whereas *M. a. paratuberculosis* was recovered from all three vials after treatment with HPC/BHI followed by VAN (Group FB).

For the tissue decontamination protocols, 72 hours in 0.75% HPC was associated with about a one log reduction in numbers (compare Group TB with Groups TE or C). The routine EMAI tissue protocol also demonstrated a marked sub-sampling effect in the removal of a 100μL aliquot from the bottom of a 30 mL tube, seen by comparison of results for Groups TA and TB. Addition of a centrifugation step in Group TB resulted in a tenfold increase in the number of viable organisms recovered. A tenfold sub-sampling effect was allowed for in the design of the experiment, so that the total reduction in numbers due to the sub-sampling in the routine EMAI protocol (Group TA) was about 1/100 which is only marginally better than the 1/300 reduction in numbers calculated on volume alone (100μL from 30 mL). In other words, sedimentation over 72 hours was not particularly effective in concentrating *M. a. paratuberculosis* in the routine tissue protocol. The increased sensitivity of the centrifugation protocol demonstrated in Experiment 1 was assessed for real tissue samples in Experiment 2. Despite a highly significant increase in contamination rates for the centrifugation method, this method detected about twice as many *M. a. paratuberculosis* positive tissues as did the routine method.
The difference is likely to be a result of numbers of organisms. The mean for CGI1000 of tissues positive by both methods was less than that for samples positive by the centrifugation method alone. All samples positive only by the centrifugation method had CGI1000 in excess of 42 days. The general relationship for CGI1000 and inoculum size established in Section 3 (Figure 2.3.7) suggests that very few M. a. paratuberculosis organisms (10 or less) are likely to be present in the inocula with such CGI1000. It is then reasonable to expect that the routine method (demonstrated in Experiment 1 to have a detection limit one log higher than the centrifugation method) would fail to detect M. a. paratuberculosis in many such samples.

Some of the areas within the decontamination protocols where loss of numbers occur are suggested by this study and may facilitate future refinements to increase sensitivity of the procedures. However, any change would need to be assessed also for increased contamination of cultures which may prevent the identification of positive samples, as was done in Experiment 2 of this study for the centrifugation method of tissue decontamination.

**General discussion**

The studies described in this chapter have significantly enhanced the ability to determine viable numbers of M. a. paratuberculosis (ovine strains) in a variety of situations. A simple technique for estimating the numbers of organisms inoculated into Bactec vials was developed, based on previous work with defined bovine strains. For a particular isolate characterised by a most probable number (MPN) estimation, simple measurement of the number of days taken for the cumulative growth index to reach 1000 (CGI1000) was shown to provide an accurate estimate of the MPN value (with no more error than that inherent in the MPN estimation). This will be particularly useful for determining numbers of organisms in experimental inocula and in laboratory manipulations of cultured organisms. A more general relationship between CGI1000 for a single Bactec vial for uncharacterised isolates of unspecified origin (from cultures, tissues, faeces or soils) was developed using results from the current study and previous MPN data generated from other research projects in the EMAI laboratory. While the prediction bands were wider (+/- 1.5 to 2 logs), this relationship will be valuable to estimate numbers of M. a. paratuberculosis from a variety of sources when only routine Bactec culture has been undertaken.

The current studies also demonstrated that -80 °C storage of suspensions of M. a. paratuberculosis was associated with minimal loss of viable numbers. This will allow the efficient storage of well characterised experimental inocula. Thus future experimental infections at defined dose levels can be done with more confidence and accuracy than was possible in the pen trials described in
Chapter 3. In these trials direct counts were used to provide an estimate of numbers, with retrospective determination of viable numbers by MPN estimations.

Both the efficient -80 °C storage and the simple enumeration techniques allowed the measurement of the effect of sample decontamination on the numbers of *M. a. paratuberculosis* finally inoculated into Bactec vials. The current studies indicated a loss of about 1 and 2 logs respectively in the standard faecal and tissue culture techniques, and allowing for sub-sampling effects, the detection limit was shown to be about $10^3$ organisms per tissue sample or $10^2$ per faecal sample. A method for tissue culture using a final centrifugation step was more sensitive (about a log better), but subject to higher contamination rates. The fact that detection limits are at least $10^2$ organisms is significant for the interpretation of culture results. We need to avoid thinking that animals which are culture-negative for *M. a. paratuberculosis* are not infected. They may indeed be uninfected, but could also be infected at a level below the detection limit for the culture technique. These enumeration techniques will also allow assessment of levels of infection in tissues or faeces, which will facilitate further insights into the pathogenesis of the disease, and also facilitate epidemiological investigations, where the levels of faecal excretion or pasture contamination are important.

These studies also emphasised some differences in the behaviour in the laboratory between the ovine strains of *M. a. paratuberculosis* (all field isolates) and the laboratory-adapted bovine strain 316V. Organisms of bovine strain took longer to reach a CGI of 1000 than corresponding numbers of ovine strains, so that separate predictive relationships would be necessary. Also, bovine strain 316V, even when prepared in PBST, vortexed and passed repeatedly through a fine gauge needle still had many large clumps, so much so that counting of individual cells in stained smears was impossible. Cultured ovine strains, on the other hand, emulsified readily in PBS even without Tween-80. If other bovine strains behave similarly to 316V, then the reported numbers of organisms used for experimental infections with bovine strains and enumerated by plate culture may be inaccurate. Each clump would be counted as a single unit and significant underestimation would be likely.
Chapter 3. Experimental infection of weaner lambs with *M. avium* subsp. *paratuberculosis* – pilot study in housed sheep

**Summary**
In controlled pen trials, lambs were given low oral doses (1.3 to 6.9 X 10^7 viable organisms) of ovine strain *M. a. paratuberculosis*. Successful infection was demonstrated by culture of tissues 7 or 14 weeks after the first dose. There were no associated gross or microscopic lesions. Only one lamb gave a positive result in the CSL ELISA for antibodies to *M. a. paratuberculosis*. Results for gamma interferon showed some correlation with infection status but lacked specificity. Skin testing with intradermal Johnin detected all three culture-confirmed infected lambs at 13 weeks, and one of three culture-confirmed infected lambs at 6 weeks with 100% specificity. This was the first demonstration of experimental oral infection with ovine *M. a. paratuberculosis* in Australian Merino sheep at low doses likely to be representative of natural infection. Detection of early infection by tissue culture might facilitate the use of naive weaner sheep as tracer animals to detect contamination of pastures with *M. a. paratuberculosis*.

**Introduction**
Eradication of ovine Johne’s disease requires destocking sheep and spelling land until *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) has died out. At the conclusion of a decontamination period, currently deemed to be 2 consecutive summers or 15 months, there is no simple way to assess either the residual level of contamination of the environment or the risk residual contamination poses to livestock. Ovine strains of *M. a. paratuberculosis* have been shown to survive for 13 months on shaded pasture, and for 7 months in exposed situations, but such studies will not determine whether infectious doses of the organism remain or whether these viable organisms are available to sheep. For example they might be caught in the soil profile and might pose no risk.

For the above reasons it was thought necessary to develop an infection model that uses live sheep. Such an approach is used in other disease control or monitoring programs, particularly for viral diseases, and the animals involved are known as tracers or sentinel livestock. The tracers or sentinels provide an early warning that an infectious disease is present or active. Johne’s disease differs from most other infectious diseases of farm livestock because the incubation period is measured in years not days or weeks. This is a major disadvantage for use of tracer animals. There is a need to find a way of detecting the infection soon after it has occurred. The tracer weaner pilot study described here was undertaken for this reason.
Work by Brotherston et al in the UK in the 1960’s using Cheviot sheep as an experimental model for bovine Johne’s disease demonstrated that single or multiple oral doses from $10^3$ to $10^9$ colony forming units (CFU) of *M. a. paratuberculosis* could produce detectable infection in intestines and MLN of most inoculated sheep as early as four weeks after the last dose.\(^{42}\) Significantly, this was many months to years before the likely development of clinical disease. Such early detection of *M. a. paratuberculosis* might be utilised to determine the infectivity of pasture following removal of infected sheep, using “tracer-weaner” lambs to pick up organisms from pasture.

This study was a pilot trial under controlled conditions. The major aim was to determine whether infection of recently weaned merino lambs with Australian ovine *M. a. paratuberculosis* isolates, at accurately enumerated doses consistent with natural exposure, could be detected by tissue culture 2 to 4 months post infection. This could also provide insights into the early pathogenesis of *M. a. paratuberculosis* infections in sheep, and help evaluate the utility of tests for delayed type hypersensitivity (DTH), gamma interferon (IFN-\(\gamma\)), humoral antibodies and faecal culture in the diagnosis of early infection.

**Methods**

**Experimental design:**

This is summarised in Table 3.1.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of lambs</th>
<th>(M)paratb per dose (intended)</th>
<th>Freq of dosing</th>
<th>Total dose (intended)</th>
<th>Clinical sampling (weeks after first dose)</th>
<th>Necropsy (weeks after first dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s: control</td>
<td>5</td>
<td>0</td>
<td>3x1 wk</td>
<td>0</td>
<td>0,2,4,6,7</td>
<td>wk 7</td>
</tr>
<tr>
<td>s: low</td>
<td>6</td>
<td>10^2</td>
<td>..</td>
<td>3x10^2</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>s: medium</td>
<td>6</td>
<td>10^4</td>
<td>..</td>
<td>3x10^4</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>s: high</td>
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<td>10^8</td>
<td>..</td>
<td>3x10^8</td>
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<tr>
<td>e: control</td>
<td>5</td>
<td>0</td>
<td>10x1 wk</td>
<td>0</td>
<td>0,2,4,6,8,10,12,14</td>
<td>wk 14</td>
</tr>
<tr>
<td>e: low</td>
<td>6</td>
<td>10^2</td>
<td>..</td>
<td>10^3</td>
<td>..</td>
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<td>..</td>
</tr>
<tr>
<td>e: high</td>
<td>3</td>
<td>10^8</td>
<td>..</td>
<td>10^9</td>
<td>..</td>
<td>..</td>
</tr>
</tbody>
</table>

\(s = \text{short regime (3 doses one week apart)} \quad \text{e = extended regime (10 doses one week apart)}\)

**Animals:**

40 Merino lambs (12-16 wk old) from the Elizabeth Macarthur Agricultural Institute (EMAI) flock (tested negative for Johne's disease by serology and faecal culture) were weaned onto wire
in the EMAI medium security animal house, drenched with Ivomec (Merial) and Sepronver (Pfizer) for internal parasites and fed ad-lib sheep pellets (mainly lucerne in composition). One week before inoculation they were allocated randomly into groups. All groups were held separately; different dose groups in different rooms, and different schedules of the same dose separated by double weldmesh partitions in the same room. Any intervention (eg routine cleaning, sampling or dosing) was done in order from the control, to low, to medium, to high dose groups. Disinfectant (Phensol, Whiteley Industries, North Sydney) footbaths were used on exit from each room, and all effluent was heat-treated at 70 °C for one hour. Lambs were euthanased with intravenous (IV) barbiturate 5 weeks after the last dose.

**INOCULA:**

*M. a. paratuberculosis* was isolated in modified Bactec medium\(^{368}\) from six separate faecal samples from sheep from a known infected farm, sub-cultured onto slopes of modified Middlebrook 7H10 medium and its identity verified by IS\(^{900}\) PCR, REA and IS\(^{1311}\) PCR.\(^{205, 369}\) Colonies were harvested into phosphate buffered saline (PBS) from these slopes, pooled and used to inoculate another Bactec vial that was used as stock culture. Subcultures into fresh Bactec vials were made from this vial when required. These were then subcultured after 3-4 weeks onto modified 7H10 slopes. Colonies from six 6-week old slopes (passage level 5) with confluent growth were harvested into a total volume of 1ml PBS. This was termed “undiluted suspension”, some of which was used immediately, and the remainder stored at 4 °C until used. Three separate undiluted suspensions were used, one prepared at week 0 for inoculations on weeks 0, 1 and 2, the second prepared at week 3 for weeks 3, 4 and 5, and the third prepared at week 6 for weeks 6, 7, 8 and 9. The total number of *M. a. paratuberculosis* (viable and non-viable) in the suspensions was determined by direct counts of appropriate dilutions in a Thoma counting chamber. Based on previous experiments (Chapter 2, Part 1) the numbers of viable *M. a. paratuberculosis* were estimated to be a half to one log lower than the direct count. Undiluted suspension was diluted in PBS immediately before each inoculation to provide the intended dose of viable organisms for each lamb in a volume of 2 ml, which was delivered to the back of the pharynx using a bulb-ended needle. The actual dose of viable organisms was determined retrospectively from the undiluted suspensions by culture in modified Bactec media using a serial dilution technique to provide most probable number (MPN) estimates, as described in Chapter 2. Because some loss of viability of *M. a. paratuberculosis* during storage in PBS at 4 °C was likely, viable estimations were repeated at weeks 2, 5 and 9 for each suspension respectively and the average number of viable organisms in each dose determined.
CLINICAL AND NECROPSY SAMPLING:

Lambs were sampled immediately before the first dose, fortnightly thereafter (immediately before any scheduled dosing) and on the day of necropsy. One faecal pellet was collected manually from the rectum of each lamb at each sampling time. Blood was collected from the jugular vein into plain and lithium heparin vacutainers. In addition faecal samples were collected daily from the high and medium dose groups (extended regime only). At necropsy 1 to 5 g samples from a range of tissues (caecum, colon, ileocaecal valve (ICV), terminal ileum (TI) x 4, jejunum - with and without Peyer's patches (PP), duodenum, ileocaecal node, caudal mesenteric node, two more mesenteric nodes, retropharangeal node and pharangeal tonsil) were collected for *M. a. paratuberculosis* isolation. The same range of tissues was also collected into 10% neutral buffered formalin for histopathology and a further replicate range of samples snap-frozen and stored in liquid nitrogen for immunoperoxidase (IPX) studies (see Chapter 5).

M. A. PARATUBERCULOSIS ISOLATION:

The fortnightly and necropsy samples were processed on the day of collection. Additional daily faecal samples were frozen at -80 °C for up to 5 months until processed. Faecal samples and tissue samples were processed and incubated using routine techniques.³⁰⁹ Bactec vials were incubated for 12 weeks, and the identity of any isolates confirmed by PCR and REA as previously described.⁷⁷ ³⁰⁹ The number of days for the cumulative growth indices (CGI) in Bactec cultures from tissues to reach 1000 was extrapolated from the weekly readings and the approximate number of viable organisms in the inoculum determined using the techniques described in Chapter 2. The culture technique used had an analytical sensitivity of about 1x10³ organisms per tissue sample, and 1x10² organisms per faecal sample (Chapter 2, Part 5).

HISTOPATHOLOGY:

Tissues were processed routinely for histopathology, sectioned at 5 μ, stained with haematoxylin & eosin (H&E) and a Ziehl-Neelsen (ZN) technique, and examined by light microscopy. In addition frozen sections were labelled for lysozyme using an IPX technique (Chapter 5). This technique is reported to increase the sensitivity for the detection of isolated, small granulomas.²⁵⁵

SEROLOGY FOR ANTIBODIES TO M. A. PARATUBERCULOSIS:

Plain blood samples were allowed to clot and retract. Serum was removed and stored at -20 °C for up to 4 months until assayed in an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Parachek Johne’s Absorbed EIA, CSL, Parkville, Victoria) according to manufacturer’s instructions.
**Intradermal Testing for Delayed Hypersensitivity:**

One week before euthanasia lambs were injected intradermally on the wool-free inner thigh with 0.1 ml of Johnin purified protein derivative (PPD) (0.5 mg/mL, CSL, Parkville, Victoria). Skin fold thickness was measured with vernier callipers before injection and 72 hours later. The averages of duplicate measurements were used, and the increase in skin-fold thickness calculated.

**Gamma Interferon Assay:**

Lithium heparin blood samples were assayed for gamma interferon (IFN-γ) using a commercial test kit (Bovigam, Bovine gamma interferon test, CSL, Parkville, Victoria) according to the manufacturer’s recommendations with slight modifications. Briefly, within 3 hours of collection three 1.5 mL aliquots of well-mixed heparinised blood were incubated for 18 hours at 37 °C in Eppendorf tubes with 100µL of bovine PPD (300 µg/mL), avian PPD (300 µg/mL) and PBS respectively. Plasma was collected after centrifugation at 500 g for 10 minutes, and stored at -20 °C for up to five months. Samples were thawed overnight at 4 °C, allowed to equilibrate to room temperature and thoroughly mixed by vortexing before use in the enzyme immunoassay (EIA). The EIA was performed exactly to the manufacturer’s recommendations. Briefly, samples were added to 96 well microtitre plates coated with antibody to bovine IFN-γ, incubated, washed, conjugate (horseradish peroxidase labelled anti-bovine IFN-γ) added, incubated, washed, enzyme substrate added, incubated, stopping solution added and the optical density (OD) read at 450 nm. Plasma was again frozen at -20 °C for a further 4 months and the EIA repeated, after centrifugation of the thawed plasma samples to remove any clots which had formed. The gamma-interferon responses were compared between groups at each sampling time with a non-parametric test (Mood’s median test, Minitab statistical software).

**Results**

An overview of all the results for each sheep is given in Table 3.2 and a full summary of results is given in Appendices 3a to 3i.
Table 3.2. Overview of results for each sheep

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Group</th>
<th>Total dose</th>
<th>IFN-(\gamma)</th>
<th>ELISA</th>
<th>Daily faecal culture</th>
<th>Skin test</th>
<th>Histological culture</th>
<th>Necropsy findings</th>
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</thead>
<tbody>
<tr>
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<td>-</td>
<td>ns</td>
<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
<td>-</td>
<td>ns</td>
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* Listed as positive if a positive result at any sampling time

* Total number of viable *M. a. paratuberculosis* (determined retrospectively from MPN estimations)

* Positive result using cut-off of 0.135 (mean + 2 standard deviations of Day 0 values)

* Positive result using manufacturer’s recommended criteria

* Positive results using a cut-off value of 0.37 mm (mean + 2 sd of control lambs)

* These lambs gave positive results before any possible *M. a. paratuberculosis* exposure

* Positive 1-6 days after dosing

* ns = not sampled  s = short dose regime  e = long dose regime
QUANTIFICATION OF M. A. PARATUBERCULOSIS DOSE:

The log_{10} direct counts and MPN estimations for each undiluted suspension used in the preparation of inocula are detailed in Appendix 3a, and the actual doses compared to the intended doses in Appendix 3b. MPN estimations for viable numbers of *M. a. paratuberculosis* in this experiment were up to 2 logs lower than the direct counts. The actual numbers of viable *M. a. paratuberculosis* organisms in each dose were about one log less than the intended dose.

CULTURE OF TISSUES FOR M. A. PARATUBERCULOSIS:

Results for each tissue are given in Appendix 3c. No positive cultures were obtained from any sheep in the control, low or medium dose groups. The organism was recovered from at least one tissue of all 6 sheep which had received the high dose level, for both short and extended dosing regimes (7 and 14 weeks after first infection). *M. a. paratuberculosis* was recovered from the TI of 4 sheep, the ICV of 3 sheep, the mesenteric lymph nodes (MLN) of 4 sheep, the tonsil of 2 sheep and from the jejunum of only one sheep. No positive cultures were obtained from any other tissue from those sheep. The contamination rate (number of cultures with positive growth index but negative for *M. a. paratuberculosis* on PCR) for these 600 tissue cultures was zero. The days taken for the CGI to reach 1000, and the calculated approximation for the numbers of viable *M. a. paratuberculosis* in each cultured sample are detailed in Appendix 3d. Highest levels of infection (about 10^5 organisms per sample) were found in two of three TI samples from sheep 35 and in the jejunum of sheep 40. Most positive intestinal samples had fewer organisms, similar to the numbers in the MLN samples which ranged from the lower detection limit of 10^3 to about 10^4 per sample. Most individual tissue samples were less than 2 gm in weight, so these values are also approximate infection levels per gram. Even assuming that only 10% of organisms given orally were taken up by the lambs, and that the tissue samples were each only 1% of the total weight of susceptible tissues, these values do not indicate a large increase in the numbers of organisms in the sheep tissues above the levels initially taken up.

FAECAL CULTURE:

A summary of all results from fortnightly samples is given in Appendix 3e and from daily samples in Appendix 3f. All cultures from the fortnightly faecal samples were negative suggesting that there was no excretion of *M. a. paratuberculosis* during the trial. Of the 240 samples cultured, five had positive growth indices due to contaminants. From the additional daily faecal samples positive results were obtained from three sheep in the high dose group on
four separate occasions, 1 to 6 days after dosing. There were six contaminated cultures from 438 daily samples. In total the contamination rate for faecal cultures in this trial was 11/678 (1.6%).

**GROSS PATHOLOGY:**

No significant gross lesions were present in any sheep.

**HISTOPATHOLOGY:**

No lesions were seen in routine H&E sections from any tissue in any sheep. ZN stained sections revealed three acid-fast bacilli (AFB) in a single macrophage in a MLN from sheep 35. Because this was an isolated macrophage and not an aggregation of macrophages, this finding was not considered to be a lesion. No other sheep had AFB in the routine sections from formalin fixed tissue, despite diligent searching. IPX for lysozyme detected a single macrophage clump in the interfollicular lymphoid tissue of the tonsil in sheep 39. Examination of serial ZN stained cryostat sections from the same block revealed a single AFB in a macrophage within the lesion. However, the sequential sections also revealed that the granuloma was centred on a piece of vegetable matter. Again this finding was not considered to be a lesion of *M. a. paratuberculosis* infection because another cause of the granuloma was readily apparent.

**SEROLOGY FOR ANTIBODIES TO M. a. PARATUBERCULOSIS:**

A summary of the results for each sampling time is given in Appendix 3g. Sheep 40 gave a positive result at week 14. No positive results were obtained for any other sheep or at any other time during this trial.

**INTRADERMAL TESTING FOR DELAYED HYPERSENSITIVITY:**

Full results are given in Appendix 3h. A positive/negative cut-off of 0.37 mm was used. This was the mean plus two standard deviations of the results for the control sheep, yielding a theoretical specificity of 97.5%. One of three high dose, short regime lambs (number 36) was positive at 6 weeks after first infection, and all three high dose, long regime lambs (numbers 38,39,40) were positive 13 weeks after first infection with skin thickness increases in excess of 2 mm. One low dose long regime lamb was also positive with a result (0.5mm) marginally above the cut-off value. All other sheep were negative. Using tissue culture as the gold standard test, the observed sensitivity of skin testing was 66% overall (100% for the lambs necropsied at week 14) and specificity was 97%. If a cut-off of 0.55 mm was used (mean plus 3 standard deviations of control values) the observed sensitivity remained unchanged and specificity was 100%.
IFN-γ ASSAY:

In the first run of the EIA, many samples, including unstimulated controls, gave very high OD values but there was an apparently random pattern. This appeared to be related to plasma clots that formed in the tubes after thawing. The spurious high values were not seen when the assay was repeated after centrifugation of the thawed plasma samples. The corrected OD values (OD (Avian PPD) – OD (PBS)) from the latter assay for each sampling time are given in Appendix 3i. Using the manufacturer’s recommended cut-off of 0.05, five of the six confirmed infected sheep were positive on at least one occasion from week 7. However, six sheep (two controls, three medium and one high dose lambs) were also positive at the initial (unexposed) sampling and at multiple time points thereafter. Another 11 culture-negative sheep were positive at least once from week 4. The overall sensitivity for this cut-off was 83%, and specificity 56%. Thus a more specific positive-negative cut-off of 0.135 was chosen (mean plus 2 standard deviations of the day 0 values). This provides a theoretical specificity of 97.5% if the test results were normally distributed. They were not (Anderson-Darling normality test, P<0.001). Using this cut-off three of six confirmed infected sheep were positive on at least one occasion from week 7. Seven uninfected sheep were also positive on at least one occasion, three of these before any possible M. a. paratuberculosis exposure. The observed sensitivity for this cut-off was 50%, and specificity 79%. There were significant differences between IFN-γ group medians for weeks 8 and 14 (high > control, P<0.05).

Discussion

Infection of weaned Merino lambs with an Australian isolate of an ovine strain of M. a. paratuberculosis was demonstrated, with total doses from about 10⁷ to 10⁸ viable organisms. A total dose of 10⁴ or less organisms did not produce detectable infection in this trial. Thus the lowest infectious dose for lambs under these experimental conditions was somewhere between 10⁴ and 10⁷ viable organisms. In retrospect a higher “medium” dose around 10⁵ organisms would have allowed better determination of the infectious dose, but the experiment was purposely biased towards the lower doses which might represent field exposure on lightly contaminated farms. Numbers of M. a. paratuberculosis in faeces from known infected sheep have previously been quantified, and levels up to 10⁹ organisms per gram demonstrated. The decontamination procedure used to isolate M. a. paratuberculosis from faecal material reduces the apparent count by at least one log (Chapter 2), so actual levels of >10⁸ organisms per gram of faeces are possible. Ingestion of gram quantities of faeces is certainly possible, especially over time, and lambs running with clinically infected sheep might reasonably be exposed to such levels, which are at least two orders of magnitude above levels demonstrated to be infectious in...
this trial. Moreover, it is possible that cultured organisms may be less pathogenic than those excreted by infected sheep so that the minimum infectious dose for sheep exposed to faecal material may be lower than that measured in this trial. Apart from a single trial in which Brotherston et al. infected lambs with $10^3$ or $10^6$ organisms, most previous experimental infections in sheep have used doses in excess of $10^9$, and often greater than $10^{10}$ organisms. At these higher levels progressive infections occur in most dosed sheep, often with rapid development of clinical disease, a situation quite distinct from that normally seen in the field. This suggests that the dose most sheep receive in natural infections is less than that usually employed for experimental infections. A similar relationship of high dose rates leading to rapid disease is seen for cattle, and again few experiments using low doses have been undertaken. In an experiment where the relatively low dose rate of $10^6$ organisms was given to calves, successful infection was demonstrated by biopsy examinations, but there were no clinical signs nor serological responses out to 20 months. When heifers were dosed with $10^6$ to $10^8$ organisms *M. a. paratuberculosis* could be cultured from ileal tissues after 4 weeks. In this experiment lambs were successfully infected with doses of about $10^7$ organisms, whereas doses below $10^4$ organisms failed to produce detectable infection. This is in contrast to Brotherston’s work in which infection was demonstrated with doses as low as $10^3$ organisms. However, there were significant differences in the methods of enumeration of the organisms between these trials. Brotherston used colony counts on solid media. Numbers of viable *M. a. paratuberculosis* obtained by this technique may be 10-fold lower than using liquid media (Chapter 2, Part 1). Moreover, the organisms used in Brotherston’s work had cultural characteristics of the bovine strain of *M. a. paratuberculosis* which has a more pronounced tendency to clump, further reducing plate counts. Thus dose may have been underestimated, and may have been in excess of $10^5$ organisms. There may also be strain differences in response to experimental infection. It is of interest that Brotherston also demonstrated that there was a considerable increase in the numbers of organisms in the intestinal tissues over the numbers originally administered. Such was not the case in the present study.

Another difference between this and other studies was the lack of specific histological changes in any of the confirmed infected lambs. Lesions consisting of clumps of epithelioid macrophages within the intestinal (particularly TI) PP have been described in lambs examined in the first few months after experimental infection. This difference may be associated with higher doses in the earlier studies, although some of the lambs with reported lesions had
received the low doses of Brotherston (above). Strain differences might also be involved, since all previous studies which have demonstrated early lesions in sheep have used organisms with cultural characteristic of the bovine strains of *M. a. paratuberculosis*. Australian Merinos might also respond differently to the mainly British breeds used in other studies. Sheep 39 in this study did have a single focus of macrophages detected in the interfollicular area of a tonsil by IPX for lysozyme. Although sequential sections showed that this lesion was foreign body-associated, an acid fast organism was detected. If this was indeed a *M. a. paratuberculosis* organism a question arises. Was the finding of the organism a matter of good luck or did the presence of a foreign body attract macrophages, some of which were already infected? The latter would seem likely since diligent searching of other ZN stained sections from this and other confirmed infected sheep found only one other infected macrophage in the MLN of sheep 35. Sheep 39 was positive by culture for *M. a. paratuberculosis* in several tissues, but not the tonsil. Given the highly focal nature of the lesion (it was not present in the initial H&E stained section examined), the lack of positive tonsil culture was not considered remarkable.

The timing of necropsy in this study was aimed at maximising the numbers of sheep from which the organism might be cultured. A number of previous studies suggest that there is a “window period”, several months after first infection, when many exposed sheep are likely to be positive by intestinal or MLN culture. *M. a. paratuberculosis* was cultured from MLN of three of four infected lambs 20 to 41 days post infection, but from none of four cultured within 18 days of infection, and a subsequent study using similar infection protocols yielded no positive cultures at 28 days. *M. a. paratuberculosis* was recovered from intestinal mucosa of sheep, 12 and 36 hours after oral infection but could not be isolated from sheep killed after 7 days. However, infections were again demonstrated in the intestinal mucosa and MLN after 28-56 days in most sheep. In the only documented study using experimental infection of sheep with low doses of *M. a. paratuberculosis* (10³ and 10⁶ CFU) the isolation rates from tissues at necropsy were highest 3½ months after first infection, with much lower rates at 5 or 9 months. Taken together these findings suggest that *M. a. paratuberculosis* is rarely isolated from the tissues of infected sheep in the first few weeks to a month after infection, whereas cultures from one to several months post infection are more likely to be positive. In sheep exposed to low doses (equivalent to natural exposure?) the numbers of sheep positive on tissue culture then decline. This “window period” may correspond to the latter part of the classical stage 2 of tuberculosis during which logarithmic growth of mycobacteria occurs within macrophages which are not sufficiently activated to destroy them.
Organisms may be inhibited by host innate immune responses soon after infection. In specific experimental studies many organisms appear degraded or lose their acid-fast staining qualities in the immediate post-infection period.\textsuperscript{106} \textsuperscript{227} It may take a month or two before organisms surviving within macrophages build up to sufficient numbers to be detected by culture. Later (3 to 4 months) developing cell-mediated immunity (CMI) may significantly limit and even reduce numbers of organisms in a large percentage of sheep. Of course, in some sheep infection will progress, and larger numbers of organisms will be present facilitating diagnosis in those particular sheep by culture or other means. The lambs in this study were just beginning to develop CMI as shown by their DTH and IFN-\(\gamma\) responses. The timing is consistent with previous experimental work in sheep in which DTH was first seen 8 weeks after experimental infection,\textsuperscript{156} and IFN-\(\gamma\) assays first returned positive results 9-18 weeks after infection.\textsuperscript{130} \textsuperscript{314} \textsuperscript{318}

The use of the skin test to assess CMI to \textit{M. a. paratuberculosis} was surprisingly successful in this study. \textsuperscript{100}\% of culture-confirmed infected sheep were detected at 13 weeks post-infection, and 66\% of culture-confirmed infected sheep overall, with 100\% specificity. In cattle, skin testing is rarely used because it has been found repeatedly to have both low sensitivity and low specificity.\textsuperscript{62} However, while most reports suggest that the sensitivity of the skin test is quite low in adult sheep,\textsuperscript{257} the specificity is reported to be high, both in the experimental situation and in the field.\textsuperscript{257} Little attention has been paid to lambs, but early experimental work suggests that skin testing can be quite sensitive soon after infection, but that the DTH response declines over time.\textsuperscript{114} It is important to note that the results in this trial were obtained for young lambs in an animal house probably with minimal exposure to environmental mycobacteria which might stimulate cross-reactivity in the skin test. Thus a relatively small increase in skin thickness (>0.55mm) could be used as a cut-off value without loss of specificity. For adult sheep in overseas work, an increase in skin thickness of >4mm has usually been used.\textsuperscript{257} All lambs in this study would have been classed as negative using those criteria.

The IFN-\(\gamma\) results also show some evidence for the development of CMI to \textit{M. a. paratuberculosis} in these lambs, the high dose group having significantly higher medians than the control group at weeks 8 and 14. Considering individual sheep and using the more specific cut-off of 0.135, three of six high dosed animals had elevated values on at least one occasion beyond week 7, and interestingly both of these were also positive in the skin test. However, seven other sheep had elevations of similar magnitude, and for three of these unrelated to any
exposure to *M. a. paratuberculosis*. This lack of specificity would limit the use of this test in these very early stages of the disease in lambs.

The use of the skin test in this trial, one week before the final sample for other immunologic tests, was also designed to provide an anamnestic stimulus for those other tests. There was little evidence for such an effect in the results. The single positive result in the final ELISA for serum antibodies in one high dose animal only is suggestive of such an effect, given that most sheep with positive serological reactions have advanced disease with severe lesions.\(^{256}\) No sheep in this trial had any lesions but no conclusions should be drawn from this isolated result. In the IFN-\(\gamma\) test, four sheep had positive results one week after skin testing. Animals 36 (week 7) and 38 (week 14) were positive on the skin test indicating that a CMI response was already present and sheep 34 had consistently shown high levels from repeated samplings. Sheep 21 from the low dose group had an elevated level only on the final sample, but again no conclusions can be drawn from this single possible anamnestic response.

It is important to reiterate that this study was looking only at the initial infection of lambs with *M. a. paratuberculosis*. Whether individual infected lambs would go on to become clinical cases, chronic asymptomatic carriers, or eliminate the organism entirely cannot be assessed from short duration trials. None the less, infection with viable *M. a. paratuberculosis* was present in sheep tissues, including extra-intestinal tissues, five weeks after the last exposure to the organism, indicating a carrier state at that time. These lambs were certainly at risk of becoming clinical or subclinical cases of Johne's disease, and a potential source of infection to other sheep. While the ultimate fate of these sheep was not important in the context of the tracer-weaner model, this study provides the basis for design of field trials to examine the utility of tissue culture in combination with tests for CMI for the early detection of natural *M. a. paratuberculosis* infection.
Chapter 4. Early detection of natural infection of sheep with *Mycobacterium avium* subsp. *paratuberculosis*

**Summary**

Culture of tissues was shown to be the most sensitive method for the detection of early infection in sheep after natural exposure to ovine stains of *M. a. paratuberculosis*. Antemortem diagnostic tests (skin testing, IFN-γ and faecal culture) had low sensitivity at this early stage of naturally acquired disease. The prevalence of infection early after exposure to continuously highly contaminated pasture was similar in sheep first exposed as neonates, as weaners or as adults. Lambs born from ewes in an infected flock became culture-positive sooner than naive lambs suckling uninfected ewes introduced to the same infected environment. These findings suggested that groups of naive sheep, used as tracer animals and tested by culture of tissues at slaughter after 6 months exposure, might be useful to assess pasture infectivity in disease control programs.

**Introduction and aims**

In pen trial experiments (Chapter 3) 100% of weaner sheep experimentally infected with $>10^7$ organisms of ovine *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) were shown to have become infected by culture of tissues 2 to 4 months later, and skin testing for delayed type hypersensitivity (DTH) to Johnin detected 66% with 100% specificity. If such early detection were also possible in naturally exposed sheep, and particularly if skin testing allowed targeting of likely-infected sheep for necropsy, then the use of tracer weaner sheep to determine the possible infectivity of pasture could be a practical option in the control of ovine Johne's disease (OJD).

There are, however, a number of foreseeable differences and potential problems in extending the tracer weaner pen model to the field. Firstly it is not possible to predict with accuracy when infection might be first acquired, nor the size of any infectious dose. Large bolus doses or repeated small doses might both occur. Availability of infectious organisms might vary with season and climate, with pasture conditions and with changes in grazing behaviour of the sheep. While contamination levels on spelled pasture can be expected to drop over time, “hot spots” such as around sheep camps, watering points, other wet areas, heavily shaded areas or hand feeding areas may have high residual levels. The chance of exposure of lambs to infective levels of *M. a. paratuberculosis* will vary from one particular field situation to another. With regard to the use of the skin test to target likely-infected sheep for necropsy, false-positive reactions...
may result from exposure of pastured sheep to a wide variety of potentially cross-reacting environmental organisms. Other effects on the ability to mount a good cell-mediated immune (CMI) response, such as intercurrent infections or general environmental stressors may further complicate the picture.

In this series of experiments I sought to determine whether the tracer concept would work in the field in environments of varying levels of infectivity, and also whether antemortem testing for specific CMI responses would allow selection of likely-infected sheep for necropsy. A supplementary aim was to further refine the sites for culture at necropsy, and also to evaluate the effects of pooling homogenates from several sites. The design also allowed comparison of the effects of age at first exposure on subsequent *M. a. paratuberculosis* infection rates.

**Methods**

**Experimental design:**

The experiments were conducted on two separate farms, both located in the southern highlands of NSW in the endemic area for OJD. The design is summarised in Tables 4.1a (Farm H) and 4.1b (Farm A). While the same methods were applied to the experimental sheep on the two farms, and a general ranking of likely levels of exposure to *M. a. paratuberculosis* was possible, there were significant uncontrolled differences (source of sheep, pasture availability, watering methods, stocking rates, infection history) which complicate direct comparison between the two farms. But the examination of the tracer concept in several different environments was considered important, if the technique is to be used in the future to examine pasture infectivity in a wide range of field situations.
### Table 4.1a. Experimental design for field evaluation of tracer weaner concept on Farm H

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<th>Group</th>
<th>Source</th>
<th>No. of sheep</th>
<th>No. killed at each level sampling</th>
<th>Exposure level</th>
<th>Age at first exposure</th>
<th>Time of first exposure</th>
<th>Sampling schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Lamb-1W</td>
<td>EMAI</td>
<td>40</td>
<td>10</td>
<td>high</td>
<td>weaner</td>
<td>0</td>
<td>2,4,6,12</td>
</tr>
<tr>
<td></td>
<td>Lamb-1C</td>
<td>EMAI</td>
<td>10</td>
<td>2</td>
<td>none</td>
<td>na</td>
<td>na</td>
<td>2,4,6,12</td>
</tr>
<tr>
<td></td>
<td>Ewe-2L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EMAI</td>
<td>30</td>
<td>10</td>
<td>high</td>
<td>adult</td>
<td>-3</td>
<td>0,3,5</td>
</tr>
<tr>
<td></td>
<td>Lamb-2L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EMAI</td>
<td>38</td>
<td>10</td>
<td>high</td>
<td>neonate</td>
<td>-3</td>
<td>0,3,5,9</td>
</tr>
<tr>
<td></td>
<td>Ewe-2W&lt;sup&gt;e&lt;/sup&gt;</td>
<td>EMAI</td>
<td>20</td>
<td>10</td>
<td>high</td>
<td>adult</td>
<td>0</td>
<td>3,5</td>
</tr>
<tr>
<td></td>
<td>Lamb-2W&lt;sup&gt;e&lt;/sup&gt;</td>
<td>EMAI</td>
<td>20</td>
<td>10</td>
<td>high</td>
<td>weaner</td>
<td>0</td>
<td>3,5</td>
</tr>
<tr>
<td></td>
<td>Lamb-2H Farm H</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
<td>high</td>
<td>prenatal?</td>
<td>-8?</td>
<td>0,3,5,9</td>
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<tr>
<td></td>
<td>Ewe-2C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>EMAI</td>
<td>10</td>
<td>0</td>
<td>none</td>
<td>na</td>
<td>na</td>
<td>3,9</td>
</tr>
<tr>
<td></td>
<td>Lamb-2C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>EMAI</td>
<td>10</td>
<td>0</td>
<td>none</td>
<td>na</td>
<td>na</td>
<td>3,9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as months relative to weaning of the lamb groups

<sup>b</sup> 20 sheep remain to be sampled at 15 and 21 months post weaning, as part of on-going research.

<sup>c</sup> These sheep were not necropsied, but used only as controls for skin testing and IFN-γ assay.

<sup>d</sup> Controls held at EMAI

<sup>e</sup> Transported immediately prior to lambing to Farm H. Group Lamb-2L are offspring of Group Ewe-2L, born on Farm H.

<sup>f</sup> Transported at weaning (3 months) to Farm H. Group Lamb-2W are offspring of Group Ewe-2W.

**Farm H:** Experiments were conducted over two successive years. The first year was essentially a pilot trial that extended the pen trial to a highly contaminated field environment. If tissue culture at necropsy could detect *M. a. paratuberculosis* infected sheep within several months of exposure, then further work was warranted. If not, there would be little point in extending field trials to lightly contaminated environments. In the second year on Farm H the effect of age at first exposure was examined, as well as comparing sheep first exposed as weaners over the two successive years.

*Exposure levels (pasture contamination):* This farm was considered to be very highly contaminated with *M. a. paratuberculosis*. Annual mortalities of up to 20% from OJD amongst older sheep were experienced, and seropositivity in the agar gel immunodiffusion test (AGID) amongst random groups of 50 to 133 clinically normal sheep 2-3 years of age ranged from 8 to 16%. Because the sensitivity of the AGID is low, and moreover, because the majority of AGID-positive sheep have the multibacillary form of OJD (and therefore likely to be heavy faecal shedders of *M. a. paratuberculosis*), these figures indicate that very high levels of environmental contamination were likely. In these trials, continuous high contamination levels were further assured by running the experimental sheep together with flocks experiencing on-going mortalities due to OJD. In Year one, experimental weaners were run with a flock of infected...
wethers and in Year 2 experimental lambs, weaners and ewes were run with a flock of infected ewes. The sheep were in large paddocks used in the routine farm enterprise. All feed was provided by the pasture and water was from streams and farm dams. As there was no pasture on Farm H considered free of contamination, control sheep were run on pasture at the Elizabeth Macarthur Agricultural Institute (EMAI). Subjective observations of prevailing pasture conditions were made at each sampling time. Pasture was classified as short, medium or long, and as green or dry. Monthly rainfall data was obtained from the owners of Farm H, an official measuring site for the Bureau of Meteorology.

**Animals:** In the first year, Merino weaners were from the OJD-free EMAI flock, and of the same source flock, genetic background and age as those used in the earlier pen trial (Chapter 3). In the second year, Merino lambs from Group Lamb-2H were the offspring of the infected ewe flock on Farm H, while Merino sheep in all other groups were from the OJD-free EMAI flock, although from a different line of sheep to those in Year 1. These groups enabled comparisons between first exposure as lambs, weaners or adults, and also with lambs born from potentially infected mothers. Group Lamb-2W also enabled a comparison with Group Lamb-1W for the effect of different years (different climate and pasture conditions).

### Table 4.1b. Experimental design for field evaluation of tracer weaner concept on Farm A

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of sheep</th>
<th>No. killed at each sampling</th>
<th>Level and duration of exposure</th>
<th>Age at first exposure</th>
<th>Time of first exposure</th>
<th>Sampling schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-High-W</td>
<td>40</td>
<td>10</td>
<td>high from weaning</td>
<td>weaner</td>
<td>0</td>
<td>2,4,6,12</td>
</tr>
<tr>
<td>A-Low-W</td>
<td>40</td>
<td>10</td>
<td>low from weaning</td>
<td>weaner</td>
<td>0</td>
<td>2,4,6,12</td>
</tr>
<tr>
<td>A-High-LW</td>
<td>32</td>
<td>8</td>
<td>high from birth and from weaning</td>
<td>neonate</td>
<td>-3</td>
<td>2,4,6,12</td>
</tr>
<tr>
<td>A-High-L</td>
<td>32</td>
<td>8</td>
<td>high from birth to weaning only</td>
<td>neonate</td>
<td>-3</td>
<td>2,4,6,12</td>
</tr>
<tr>
<td>A-Control</td>
<td>20</td>
<td>5</td>
<td>none</td>
<td>na</td>
<td>na</td>
<td>2,4,6,12</td>
</tr>
</tbody>
</table>

- These sheep shared the same pasture after weaning.
- These sheep shared the same pasture from birth until weaning.
- Expressed as months relative to weaning

**Farm A:** Field aspects of experiments on Farm A were conducted concurrently with those in Year 1 on Farm H, but samples collected at necropsy were stored, and processed later, after the first positive culture results from Farm H became available. The effects of two different levels of contamination were examined, as well as the effect of age at first exposure (weaner or neonate).

**Exposure levels (pasture contamination):** Farm A had a past history of low prevalence OJD infection but unlike Farm H, mortalities had never occurred. A series of small paddocks (5 to 10 ha) with pasture of varying contamination levels had been prepared as part of other research and were

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available for use in this trial. Occasional supplementary feeding was necessary and water was provided from troughs. Control pasture, considered free of *M. a. paratuberculosis* contamination, had carried no infected sheep for at least 18 months and had been grazed by cattle in the interim. Low contamination pasture had been grazed by introduced infected ewes for two months only, commencing 3 months prior to weaning, so that pasture was again free of infected sheep for one month prior to the introduction of experimental weaners. High contamination pasture had been grazed by introduced infected ewes for six months, commencing four months prior to weaning. Thus Group A-High-LW was running with infected ewes from birth until 2 months post-weaning, Group A-High-L from birth until weaning and Group A-High-W from weaning until 2 months post-weaning. The infected ewes used for these contaminations had been specifically purchased from another heavily infected property, and were from a flock experiencing annual OJD mortalities of up to 10% and with a seropositivity rate of 12.7%. In contrast to the situation on Farm H where pasture contamination levels were high and continuous, pasture contamination levels on Farm A were likely to be dropping over time because the infected adults were removed soon after weaning. In previous studies a 2 log reduction in numbers of viable *M. a. paratuberculosis* on pasture occurred over a 10 week period.\(^{375}\)

*Animals:* All experimental lambs and weaners were surplus offspring from a flock of OJD-free Merino ewes purchased specifically for other OJD research on Farm A. They were unlikely to be of the same genetic line as those used on Farm H.

**Intradermal testing for delayed hypersensitivity (DTH):**

All surviving sheep were tested at each sampling as detailed in Tables 4.1a and 4.1b. In addition, the ewes from Year 2 were tested about 3 months before and again immediately prior to lambing. Sheep were injected intradermally on the wool-free inner thigh with 0.1 mL of Avian tuberculin purified protein derivative (PPD) (25,000 IU/mL, CSL, Parkville, Victoria). Skin fold thickness was measured with vernier callipers before injection and 72 hours later. At the 6 month sampling sheep from Groups Lamb-1W and Lamb-1C were tested concurrently on the opposite hind leg with 0.1 mL of Johnin PPD (0.5 mg/mL, CSL, Parkville, Victoria). Positive-negative cut-off points were established using all available age-matched unexposed pastured sheep from both farms and both years of the trial. The results (mean plus 2 standard deviations, giving a theoretical specificity of 97.5%) for tests on unexposed young sheep of ages 0-6 months (n = 201), 7-12 months (n = 58) and greater than 12 months (n = 18) respectively were pooled to provide the respective cut-off values.
**Gamma interferon (IFN-γ) assay:**

This was performed on samples from Year 2 only. Blood was collected from the jugular vein into lithium heparin vacutainers, and held at room temperature for less than 12 hours prior to processing using a commercial test kit (Bovigam, Bovine gamma interferon test, CSL, Parkville, Victoria). The protocol used differed slightly from that used in the pen trial (Chapter 3). For each sample three 1.5 mL aliquots of well mixed blood were incubated for 18 hours at 37 °C with 100 µL of Johnin PPD (300 µg/mL), Avian PPD (300 µg/mL) or phosphate buffered saline (PBS) in polystyrene cell culture plates (Costar, Corning International, New York). Plasma was collected after centrifugation at 500 g for 10 minutes, and transported overnight at 4 °C to CSL for the enzyme immunoassay (EIA). Results were assessed using the manufacturer’s recommended criteria. A response to Avian PPD was recorded if optical density (OD) (Avian PPD) was > 0.05 above that for PBS (equivalent to results from Chapter 3). Similarly a response to Johnin was recorded when OD (Johnin) was > 0.05 above that for PBS. Only when OD (Johnin) was > 0.05 above both OD (PBS) and OD (Avian PPD) was the result was recorded as positive. This latter criterion was recommended by the manufacturer in an attempt to avoid false positive results.

**Necropsy sampling:**

At each sampling period sheep were selected for necropsy on the basis of the skin test results obtained immediately prior to necropsy. Half of the sheep selected from each group had the largest increases in skin thickness and half had the smallest increases. Sheep were euthanased with intravenous (IV) barbiturate. Enlarged mesenteric lymph nodes (MLN), thickening of terminal ileum (TI), or mesenteric lymphangitis were recorded as possible gross lesions of OJD. A range of tissues (ileocaecal valve (ICV), TI (5 g pool of 3 samples from last 300 mm of ileum), MLN (5 g pool from three nodes) and tonsil (pool from both sides) were collected into sterile containers and transported at 4 °C for *M. a. paratuberculosis* isolation. Duplicate samples from adjacent tissue were collected into 10% neutral buffered formalin for histopathology. Faeces were also collected for *M. a. paratuberculosis* isolation from selected samplings in Year 1 and from all necropsied sheep in Year 2.

**M. a. paratuberculosis isolation:**

Tissue samples from Farm H, Year 1 (2, 4 and 6 month samples) were processed within 24 hours of collection by standard tissue culture techniques. Tissue samples for all other groups were frozen at -80 °C within 12 hours of collection for up to 12 months until processed using a final centrifugation step as previously described (Chapter 2). All faecal samples were frozen at -
80 °C for up to 12 months until processed routinely. Bactec vials were incubated for 12 weeks, and the identity of any isolates confirmed by IS900 PCR and REA. Gram-stained smears were prepared from selected growth index (GI) positive Bactec vials which were also subcultured onto modified 7H10 Middlebrook agar, with and without Mycobactin J to characterise contaminating organisms.

Culture of pooled homogenates: During the processing of tissue samples 1 mL aliquots of tissue homogenates from each of TI, ICV and MLN were removed, pooled and frozen at -80 °C. Selected pooled homogenates were later processed using the centrifugation method. Pooled homogenates from the 9 month sampling in Year 2 were not frozen, but processed concurrently with the individual tissues samples. The ability to detect infected sheep by culture of individual tissues or by culture of pooled homogenate was compared using McNemar’s Chi-square test for paired observations. Culture using individual tissues was considered positive in this analysis if any of TI, ICV or MLN was positive. The contamination rates for cultures of individual tissues and pooled samples were compared using the Chi-square test on 2 x 2 contingency tables. A tissue sample was considered contaminated if it had a positive growth index in Bactec culture but was negative for M. a. paratuberculosis by IS900 PCR. Contamination which did not prevent M. a. paratuberculosis identification was ignored in this analysis.

Histopathology:

Tissues were processed routinely for histopathology, sectioned at 5 µM, stained with haematoxylin and eosin (H&E) and a Ziehl-Neelsen (ZN) technique, and examined by light microscopy. H&E stained sections were examined routinely for lesions, scanning first under lower powers, then focusing on suspicious areas under higher magnification up to 400 X. For ZN stained sections particular attention was focused on any suspicious areas identified in H&E sections. These were examined thoroughly under 400 X magnification and any fields containing material suspicious of being acid-fast bacilli (AFB) were examined under oil-immersion. Sheep were classified as histologically positive for Johne's disease when typical focal, multifocal or diffuse lesions consistent with OJD were present in the H&E sections. The least pathology recorded as positive was the finding of at least two clumps of macrophages with typical epithelioid morphology and no other cause apparent in a usual predilection site, whether or not AFB were present. An equivocal result was recorded when no AFB were detected in ZN stained sections, and isolated clumps of cells with morphology suggestive, but not completely typical of the macrophages usually associated with OJD, or granulomas suggestive of OJD, but more consistent with another aetiology, were present in H&E stained sections. Macrophages were considered atypical if they contained refractive or pigmented material, and granulomas
were considered more consistent with another aetiology if they had mineralised contents, contained material suggestive of vegetable matter or the possible remnants of parasites (such as helminths or coccidia), or if there were large numbers of eosinophils within the granuloma.

**Results**

A summary of the group results for all trials is given in Tables 4.2a (Farm H) and 4.2b (Farm A). Pasture and rainfall data is shown in Figure 4.1. Detailed results for each sheep are summarised in Appendices 4a (Farm H, Year one), 4b (Farm H, Year 2) and 4c (Farm A).

**Figure 4.1: Rainfall and pasture conditions during Years 1 and 2 of field exposure on Farm H**

<table>
<thead>
<tr>
<th>Year</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year 2</td>
<td>-3</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>

**Pasture conditions:**
- Year 1: long dry, short dry, med green, short green
- Year 2: - long short, short dry, med green, short green
Table 4.2a. Tracer weaner field evaluation on Farm H - summary results

<table>
<thead>
<tr>
<th>Group</th>
<th>Age at first exposure</th>
<th>Time of necropsy</th>
<th>Culture +ve&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Histo +ve&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Skin test +ve&lt;sup&gt;c&lt;/sup&gt;</th>
<th>IFN-g +ve&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>age at necropsy mths after weaning mths after exposure</td>
<td>no. of sheep (%)</td>
<td>no. of sheep (culture +ve)</td>
<td>no. of sheep (culture +ve)</td>
<td>no. of sheep (culture +ve)</td>
<td>no. of sheep (culture +ve)</td>
</tr>
<tr>
<td><strong>Year 1</strong></td>
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<td></td>
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</tr>
<tr>
<td>Lamb-1W</td>
<td>weaner</td>
<td>5 2 2</td>
<td>0 (0) ns</td>
<td>0 (0)</td>
<td>5 (0) ns</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>7 4 4</td>
<td>0 (0) 0</td>
<td>0 (0) 2 (0) ns</td>
<td>0 (0)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 6 6</td>
<td>2 (20) 0</td>
<td>0 (0) 0 (0) ns</td>
<td>0 (0)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 12 12</td>
<td>6 (75) 2 (2)</td>
<td>2 (2) 2 (1) ns</td>
<td>2 (1)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Lamb-1C</td>
<td>na</td>
<td>5 2 na</td>
<td>0 (0) ns</td>
<td>0 (0)</td>
<td>0 (0) ns</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>7 4 na</td>
<td>0 (0) 0 ns</td>
<td>0 (0) 1 (0) ns</td>
<td>1 (0)</td>
<td>ns</td>
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</tr>
<tr>
<td></td>
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<td>0 (0) ns</td>
<td>0 (0) 1 (0) ns</td>
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</tr>
<tr>
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<td>15 12 na</td>
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<td>0 (0) 0 (0) ns</td>
<td>0 (0)</td>
<td>ns</td>
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</tr>
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<td><strong>Year 2</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ewe-2L</td>
<td>adult</td>
<td>0 3</td>
<td>0 (0) 2 (0) 0 (0)</td>
<td>2 (0) 0 (0)</td>
<td>2 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td>3 6</td>
<td>1 (10) 0 0</td>
<td>0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
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<tr>
<td></td>
<td>adult</td>
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<td>1 0 (0) 0 (0)</td>
<td>2 (1)</td>
<td></td>
</tr>
<tr>
<td>Lamb-2L</td>
<td>neonate</td>
<td>3 0</td>
<td>0 (0) 0</td>
<td>0 (0) 3 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 3 6</td>
<td>1 (10) 0</td>
<td>0 (0) 5 (0)</td>
<td>0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 5 8</td>
<td>1 (10) 0</td>
<td>0 (0) 2 (0)</td>
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<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 9 12</td>
<td>3 (43) 2 (2)</td>
<td>2 (2) 4 (3)</td>
<td>1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ewe-2W</td>
<td>adult</td>
<td>3 3</td>
<td>0 (0) 0</td>
<td>0 (0) 1 (0)</td>
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<tr>
<td></td>
<td>adult</td>
<td>5 5</td>
<td>2 (20) 0 0</td>
<td>0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td>Lamb-2W</td>
<td>weaner</td>
<td>6 3 3</td>
<td>0 (0) 0</td>
<td>0 (0) 4 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 5 5</td>
<td>2 (20) 0</td>
<td>0 (0) 0</td>
<td>0 (0) 2 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td>Lamb-2H</td>
<td>prenatal?</td>
<td>3 0 &gt;3</td>
<td>3 (30) 0</td>
<td>0 (0) 3 (1)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 3 &gt;6</td>
<td>2 (20) 0</td>
<td>0 (0) 3 (1)</td>
<td>0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 5 &gt;8</td>
<td>3 (30) 2</td>
<td>0 (0) 4 (2)</td>
<td>0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 9 &gt;12</td>
<td>6 (60) 2</td>
<td>3 (3) 5 (5)</td>
<td>1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ewe-2C</td>
<td>na</td>
<td>na 3, 9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>na ns 0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ns 4 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamb-2C</td>
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<td>na ns 0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ns 1 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> +ve in any of up to 5 separate tissue cultures (MLN, TI, ICV, tonsil, +/- pool)

<sup>b</sup> Sheep with typical lesions (+/- AFB) are listed as +ve

<sup>c</sup> Number of sheep +ve on at least one occasion prior to necropsy

<sup>d</sup> Number of sheep +ve on at least one occasion prior to necropsy; (OD (Johnin) exceeds both OD (control) and OD (Avian PPD)) by > 0.05

<sup>e</sup> Time when sampled; these sheep were not necropsied; faecal culture results are from pooled cultures for the whole group.

ns Not sampled

na Not applicable
**Overview of findings on Farm H:**

*Sheep first exposed as weaners*: In Year 1 there were no positive cultures from weaners (Group Lamb-1W) necropsied 2 or 4 months after exposure. At 6 months 20% were tissue culture-positive, and at 12 months 75% were tissue culture-positive. In Year 2, results for comparable weaners (Group Lamb-2W) were similar, with no culture-positive sheep 3 months after exposure but 20% culture-positive at 5 months.

*Sheep first exposed as neonates (Group Lamb-2L)*: Results were similar to the above with respect to time after first exposure, with no culture-positive sheep among those necropsied 3 months post-exposure (i.e. at weaning, or 3 months old). A single culture-positive lamb was first detected after 6 months exposure, and another at 8 months. By 12 months 43% were culture-positive.

*Sheep first exposed as adults*: The results were also broadly similar to those for weaners and neonates. No culture-positive sheep were detected until 5-6 months after first exposure. A single ewe from Group Ewe-2L was culture-positive in February (six months post-exposure), and two ewes from Group Ewe-2W were culture-positive in May (five months post-exposure). Exceptionally, at that same May sampling 60% of ewes from Group Ewe-2L were culture-positive, eight months post-exposure. One of these ewes was also faecal culture-positive.

*Lambs born from potentially infected ewes (Group Lamb-2H)*: These results followed a different pattern from those of the introduced naive sheep above. 20 to 30% of necropsied lambs were culture-positive at each of the samplings at 3, 6 and 8 months of age, and 60% were culture-positive at 12 months. Four of the tissue culture-positive lambs were also faecal culture-positive.

Results for histopathology, skin testing and IFN-γ are discussed in detail in the relevant sections below. Briefly, some sheep from all age classes had histological lesions of OJD, but never before 8 months of potential exposure to *M. a. paratuberculosis*. False positive skin test results were frequent at the early samplings. Few sheep had positive IFN-γ results. IFN-γ-positive lambs were also culture-positive, but IFN-γ-positive ewes were often culture-negative.
Table 4.2b. Tracer weaner field evaluation on Farm A - summary results

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<th>Group</th>
<th>Exposure</th>
<th>Necropsy mths after weaning</th>
<th>Necropsy mths after exposure</th>
<th>Culture +vea</th>
<th>Tissues Faeces (also culture +ve)</th>
<th>Histologically +veb</th>
<th>Skin test +vec</th>
<th>no. of sheep</th>
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a +ve in any of up to 5 separate tissue cultures (MLN, TI, ICV, tonsil, +/- pool)
b Sheep with typical lesions (+/- AFB) are listed as +ve
c Number of sheep +ve on at least one occasion prior to necropsy
d Number of sheep +ve on at least one occasion prior to necropsy; (OD (Johnin) exceeds both OD (control) and OD (Avian PPD) by > 0.05)
ns Not sampled
na Not applicable

Overview of Findings on Farm A:

Sheep first exposed as weaners: There were no positive tissue cultures from sheep necropsied 2 or 4 months after first exposure. At 6 months 20% of the high exposure group (A-High-W) were tissue culture-positive, and 11% were positive at 12 months. For the weaners on low contamination pasture, there was a single culture-positive result at the 12 month sampling.

Sheep first exposed as neonates: Again, these results were similar to those of sheep first exposed as weaners when interpreted with respect to length of exposure. The first positive tissue culture results were found 7 months post-exposure (7 months of age, 4 months post-weaning) when 25% of sheep necropsied from Group A-High-L (exposed from birth until weaning only) were positive, but there were no further culture-positive sheep detected in this group at subsequent samplings. In Group A-High-LW (exposed from birth through weaning) there were no culture-positive sheep detected at 5 or 7 months post-exposure, but 25% and 12.5% respectively of
sheep necropsied after 9 and 15 months exposure (6 and 12 months post-weaning) were tissue culture-positive.

None of the experimental sheep from Farm A had detectable histological lesions at necropsy. False positive skin test results were also a feature on this farm, mainly from the highly exposed groups, and no culture-positive sheep were detected by the skin test. No sheep was faecal culture-positive at the 12 month sampling.

Culture of M. a. paratuberculosis:

In total, 47 of 324 (14.5%) sheep with possible exposure to M. a. paratuberculosis were confirmed infected after necropsy and culture of tissues. 29 control sheep were also cultured and all were culture-negative. Five of 207 exposed sheep were positive by faecal culture. All were from Farm H; one a ewe 8 months after first exposure, and four lambs from Group Lamb-H at 8 or 12 months of age. All faecal culture-positive sheep were also tissue culture-positive (sensitivity of 5/41 (12.2%) with 100% specificity compared to tissue culture).

Comparison of culture of individual tissues and pooled homogenates: Samples from 145 sheep for which the pooled homogenate had been frozen prior to culture were examined. Of 21 culture-positive sheep, 10 were detected by both methods, nine were detected only by culture of individual tissues, compared to just two which were detected only by culture of pooled homogenate (Chi-square = 4.45, P < 0.05). 44/145 (30.3%) of these pooled cultures were contaminated, compared to 65/435 (14.7%) of individual tissue cultures (Chi-square = 10.88, P < 0.001). Samples from 17 sheep for which the pooled homogenate was not frozen were also examined, and there were no significant differences in detection rates or contamination rates for the individual compared to pooled cultures. Eight infected sheep were detected by culture of individual tissue samples, and these eight and an additional sheep were detected by culture of the pooled homogenate. 5/51 (9.8%) of the individual tissue cultures and 2/17 (11.8%) of the pooled cultures were contaminated.

Intradermal testing for delayed hypersensitivity:

Results for unexposed sheep are summarised in Figure 4.2, indicating a trend for greater skin thickness increases after intradermal Avian PPD with increasing age. The cut-off points established for young sheep were 0.54 mm (0-6 months), 0.98 mm (7-12 months) and 1.9 mm (> 12 months). For adult sheep the corresponding cut-off was 4.23 mm (n = 117), but the generally accepted value of 4.0 mm was used.257 Using these age-related cut-off values, 13 of 47 culture-positive sheep were detected by the skin test (observed sensitivity 13/47 or 28%).
of 306 culture negative sheep were skin test negative (observed specificity 263/306 or 85.9%). No difference was seen between the responses to Avian PPD or Johnin in the 23 lambs from Groups Lamb-1W and Group Lamb-1C tested 6 months after weaning, but there were no positive skin tests from these groups at this sampling for either preparation.

Figure 4.2. Skin-fold thickness increase 72 hours after intradermal Avian PPD in pastured sheep unexposed to Mycobacterium avium subsp. paratuberculosis

IFN-γ:

Of the samples taken 3 days prior to necropsy three were positive using the manufacturer’s revised specific criteria, and these sheep were also culture-positive (all after at least 8 months of potential exposure). Another four ewes gave a positive IFN-γ result 2-3 months prior to necropsy. All were culture negative and three of those positive IFN-γ results were obtained before any known exposure to M. a. paratuberculosis. As there were 30 culture-positive sheep from a total of 147 necropsied in Year 2, the observed sensitivity of IFN-γ testing was 10% (3/30), and the observed specificity was 97% (113/117). When considering reactions to Avian PPD in isolation (equivalent to the IFN-γ testing in Chapter 3), 13 ewes and 2 lambs showed reactivity on at least one occasion, 9 of these in the absence of any known M. a. paratuberculosis exposure. Sensitivity and specificity were 7% (2/30) and 89% (104/117) respectively.
Considering reactions to Johnin alone, 18 ewes and 5 lambs reacted on at least one occasion, 10 in the absence of any exposure, with sensitivity and specificity of 27% (8/30) and 87% (102/117) respectively.

**Histopathology:**

Eight of a total of 324 potentially exposed sheep had definite histological lesions of OJD, and all were confirmed as infected by culture of *M. a. paratuberculosis* from tissues. All were from Farm H. Seven were lambs, 12 to 15 months old, potentially exposed to *M. a. paratuberculosis* for at least 12 months, and one was a ewe exposed for 8 months. Five sheep (four lambs and one ewe) had focal lesions only in MLN, two with small numbers of AFB. Two lambs had focal or multifocal lesions only in the TI (both AFB negative). A single lamb had diffuse locally extensive lesions in one only of three TI sections with very large numbers of AFB. This lamb also had focal lesions with small numbers of AFB in the MLN. A further 56 sheep (including three controls) had equivocal lesions, but only seven of these (12.5%) were confirmed as infected by culture at necropsy, about the same number as would be expected by chance (overall, 47/353 (13.3%) of necropsied sheep were culture-positive).

**Discussion**

In this series of experiments, conducted on two independent farms, infection with *M. a. paratuberculosis* was detected by tissue culture in at least one naive introduced sheep from every potentially exposed group, 5 to 15 months after first exposure. In the heavily contaminated environment on Farm H, 43 to 75% of sheep sampled after 8 months of exposure were culture-positive, whereas in the low contamination paddock on Farm A, a single infected sheep was detected 12 months after first exposure. The cultural findings reinforce those from the earlier pen trials (Chapter 3) and from other reported studies in experimentally infected sheep,\(^{42}\) that culture of tissues is the most sensitive method currently available for the detection of early *M. a. paratuberculosis* infection. The current study is, in addition, the first demonstration of tissue culture as a practical tool for the detection of early infection in sheep after natural exposure to ovine stains of *M. a. paratuberculosis*.

Few of the sheep positive by tissue culture were detected by the ante-mortem tests for infection. Skin testing for DTH detected only 28% of infected sheep with 86% specificity, IFN-γ testing detected only 10% with 97% specificity, and faecal culture detected only 12% with 100% specificity; (the relatively low sensitivities for these tests in this study reflect their use in the very early stage of the disease process). Nor were histological lesions, often used as the “gold standard” for *M. a. paratuberculosis* infection in sheep, frequent. Only 17% of culture-
positive sheep had typical histological lesions, and all sheep with such lesions were culture-positive. Unequivocal lesions of OJD were not seen in sheep with less than 8 months of exposure. This lack of lesions in the early months after first infection is consistent with the findings for weaners experimentally infected with an ovine strain of *M. a. paratuberculosis* in pen trials (Chapter 3). This is in contrast to results commonly reported for experimental infection in sheep with high doses of bovine strains of *M. a. paratuberculosis*, which describe lesions in the first months after infection in some sheep. Such high-dose, bovine-strain infection scenarios may not be representative of the usual infection of sheep in natural situations, so that data generated from such trials, eg. the performance of diagnostic tests, should be viewed with caution. The positive histopathological findings from the present trial, although few, also provide some additional insight into the development of lesions in sheep. Most of the lesions were focal in distribution, with few or no AFB present – typical of the early lesions described by others.

However, one 15 month old lamb, potentially exposed for 12 months, had lesions which were typical of the diffuse multibacillary form of OJD in only one of three ileal sections. Such pathology is usually associated with sheep having advanced disease, and is usually expected to be widespread in the intestine. This lamb had no gross lesions, was clinically normal, and was negative on the skin test and faecal culture. The histopathological findings in this lamb suggest that in some sheep, focal lesions may progress directly to the multibacillary form, rather than gradually spread to be multifocal, and only later become multibacillary in character.

The difference in the pattern of culture results between sheep born from an infected ewe flock, in which 20-30% of sampled lambs were tissue culture-positive at 3, 6 and 9 months of age, and introduced naive sheep, suggests the possibility of transmission of infection from infected ewes to their offspring. This trial was not designed to specifically examine the details of such transmission. Congenital infection is a possibility, and has been demonstrated frequently in cattle, and once in sheep. Similarly, infection via the milk, and particularly colostrum is possible. Faecal-oral transmission is also likely to be enhanced (eg. by exposure to faecally contaminated udders), and lambs of infected ewes may be exposed to higher numbers of *M. a. paratuberculosis*. Whatever the mechanism, it appears that lambs born from an infected flock become tissue culture-positive sooner than naive lambs suckling uninfected ewes introduced to the same infected environment. This could be important in the design of disease control programs and warrants further investigation.
These experiments also suggest that age has little effect on the early establishment of infection, since neonates, weaners and ewes on Farm H had similar infection levels after similar periods of exposure. Group Ewe-2W and their lambs (Group Lamb-2W) had exactly the same exposure history, having been moved to Farm H at weaning in December, so are directly comparable. Their infection rates were identical. Similarly Group Ewe-2L and their lambs (Group Lamb-2L) were both exposed from the time of lambing on Farm H in September. Infection rates for these two groups were broadly similar to previous ones when considered relative to length of possible exposure, despite the three-month asynchrony in terms of season. However, Group Ewe-2L was remarkable in that 60% of ewes examined 8 months post-exposure were infected, compared to only 10% of their lambs after an identical exposure history. This difference may reflect the greater likelihood of grazing ewes to pick up an infectious dose off pasture as compared to their suckling lambs, and/or the effect of lactational stress on the ewes' resistance to the establishment of infection. On Farm A, weaners and neonates also had similar infection rates. Together, these findings reinforce early experimental studies with bovine strains which demonstrated similar levels of tissue infection 2½ months post infection in sheep from 3 weeks to 20 months of age. Published studies suggest that infection of older animals, however, is less likely to progress to clinical disease. The short duration of the current work does not allow any such assessment. But some speculation concerning the likely outcome of such infections in young sheep is possible. In the highly contaminated environment of Farm H a total of 21/35 (60%) of sheep examined 8 to 12 months after first exposure were found to be infected, a figure well in excess of the proportion of adult sheep which later would succumb to OJD in the normal course of events on this farm. In related on-going studies on Farm H, only 32% of 145 three year old ewes were culture-positive at necropsy (Lambeth et al, unpublished), and mortalities never exceeded 20%. This suggests that recovery or latent infection may be occurring in many infected sheep.

The results from these trials also suggest that the establishment of infection in sheep may be an on-going process, rather than primarily infection as neonates which then gradually progresses with age. Contrast the findings for sheep in the continuously highly contaminated environment of Farm H with those on Farm A where exposure levels were likely to be high initially but dropping over time. At the final samplings from Farm H higher proportions of sheep were culture-positive than at previous examinations, whereas on Farm A levels were similar or decreasing.
On Farm H, the highest numbers of sheep were culture-positive at samplings in September and December, which suggests the possibility of a climatic effect on the likelihood to pick up an infectious dose following exposure to short green pastures in winter/spring. However these experiments were not designed to look specifically at seasonal effects and such suppositions are confounded by both age and length of exposure. The rainfall and pasture conditions were also markedly different across the two years of the trial. In Year 1 pasture was long and dry at weaning (December) and dry conditions prevailed over summer. In Year 2 pasture was short and green at weaning, and continued green and lush throughout a wet summer. Despite these differences the culture results for the comparable groups first exposed at weaning (Lamb-1W and Lamb-2W) were very similar. An alternate explanation may be that immunosupression following dietary stress in late winter might facilitate the establishment of initial infection or possibly unmask previously undetected latent infection, but again this cannot be assessed from these trials. However, consider again the exceptional results for Group Ewe-2L for which 60% of sheep examined in May, 8 months post-exposure, were culture-positive. These sheep were transported to Farm H at lambing, and so were initially exposed to short green spring pasture at the same time as under lactational stress.

As discussed in Chapter 3, there may be a “window period” several months after first infection, when the greatest numbers of sheep have detectable *M. a. paratuberculosis* infections in their tissues. In the field situation it is impossible to predict accurately when such infection might be acquired, so a range of sampling times up to one year after first possible exposure was used for this series of experiments. The selected times were based on experience from the pen trials in which positive cultures were obtained 2 and 4 months after first exposure to *M. a. paratuberculosis*. In planning these field trials infection was thought more likely to occur soon after potential exposure rather than later, especially on Farm A where pasture contamination was likely to decline over time since infected sheep had been removed. Thus sampling was biased towards the first six months after exposure. The final 9 to 12 month sampling was added to make some allowance for possible seasonal variations (sheep would have experienced a full year in the contaminated environment) and to possibly provide time for the progression of lesions in some sheep. On highly contaminated pasture there was also the possibility that many sheep could have been culture-positive at early samplings and a final sampling after 12 months would then have indicated the persistence or otherwise of such early infections. A brief discussion of the way sheep were selected for necropsy is also warranted. Within each group at each sampling time, sheep were selected on the basis of having the largest or smallest DTH
reactions in the test immediately prior to slaughter. This non-random selection technique was
chosen in an attempt to determine whether the skin test would be useful as a predictor of tissue
culture status, as suggested by the results of the pen trial (Chapter 3). Especially on the pastures
with possibly low levels of *M. a. paratuberculosis* contamination, few culture positive sheep were
expected, and to make best use of the “window period”, sheep needed to be cultured as soon
as possible after a positive skin test. But the selection technique may have biased the results. If
indeed DTH is a useful predictor of culture status, then the successive removal of DTH
positive sheep at each sampling could bias culture-positive results towards the earlier samplings.
Conversely, sheep with the highest DTH levels may be the more resistant animals,112 and more
culture-positive sheep might then be expected at later samplings.

Previous studies of DTH responses to Johnin in both cattle and sheep have indicated that
repeated testing at one to 3 month intervals did not result in sensitisation of the animals.112 114 182
On the other hand, local desensitisation soon after intradermal testing has been described in
cattle but was almost eliminated by one month and no longer apparent by 2 months,182 so a
minimum 2 month interval between testing was chosen for these trials. Johnin PPD was not
available in sufficient quantity for use throughout these trials so Avian PPD was used instead.
Johnin and Avian PPD are reported to differ in that Johnin contains cellular components due
to bacterial lysis in one month old cultures in addition to secreted proteins.317 However, reports
suggest that there is little difference in sensitivity when the preparations are used to elicit DTH
in the field. Similar numbers of sheep responded to Johnin or Avian PPD following
experimental infection with *M. a. paratuberculosis*, although the intensity of reaction was greater
to Avian PPD in many sheep.156 Johnin may, however, be more specific than Avian PPD, with
only 2/6 *M. avium*-infected animals showing DTH to Johnin whereas 5/6 reacted to Avian
PPD.156 The small amount of Johnin available in the present trials was used for a limited
comparison with Avian PPD on Farm H at the 6 month sampling. Unfortunately all tests
using either preparation were negative (including 2 culture-positive sheep) so no conclusions
could be drawn. Most skin test positive sheep from samplings 2 to 4 months after first
exposure were culture-negative for *M. a. paratuberculosis*. This is in marked contrast to results
obtained in the pen trial (Chapter 3) when no false positive DTH responses were found (100%
specificity using the same cut-off values). The reasons for false positive results in the field trials
might include the lower specificity of Avian PPD as discussed above, combined with possible
cross-sensitisation by other environmental antigens. Other mycobacteria were indeed detected
in some contaminated cultures of intestine and MLN over several time periods on all farms,
although such isolations were not correlated with positive DTH in individual sheep; (in the pen trials (Chapter 3) no tissue cultures were contaminated). Interestingly, however, most of the positive DTH results from lambs in these field trials were from the most heavily exposed groups, suggesting that many of the observed early DTH responses could indeed be specific responses to *M. a. paratuberculosis* exposure. Exposure to *M. a. paratuberculosis* in numbers below those in the earlier pen trials, but above some threshold level for DTH responses might have resulted in levels of infection in the tissues below the detection limit of the culture system, or focal infection in unsampled tissues may have been missed. Another possibility is that CMI responses in pastured sheep may be more effective (perhaps via concurrent immunostimulation by other organisms) and early infection was controlled. At the later sampling times false positive reactions were less frequent with 75% of skin test positive sheep necropsied after 12 or 15 months of potential exposure confirmed infected by tissue culture. Overall the sensitivity of the skin test in detection of these early infections was also low (28%), again in some contrast to the results from the experimentally infected housed lambs. Possible reasons for these false negative results might include the possibility of lower specific DTH responses in pastured sheep due to various intercurrent immunosuppressive effects (eg concurrent infections, climatic or dietary stresses). It may also be a matter of timing. In experimentally infected sheep, DTH has been shown to develop about 2-3 months after exposure, with levels declining after some months in many sheep. Some trial sheep may have had the potential for DTH responses at some time after the 6 month testing which then declined to undetectable levels by the 12 month testing, and there was no test done in between. It is also possible that some sheep had infection at a level sufficient for detection by culture but insufficient to stimulate a DTH response. Previous experimental studies have shown that some sheep with the highest levels of infection may not show positive DTH reactions, and that the largest DTH reactions were seen in sheep with the least levels of subsequent infection. Similar observations have been recorded for other measures of CMI such as IFN-γ. Experimentally sheep with the highest IFN-γ responses were less likely to be infected at later necropsy than those with lower responses.

The tissues selected for culture in these field trials were a subset of those used in the pen trials (Chapter 3), and chosen so that had the selected subset been used on the housed sheep, no infected sheep would have been missed. In these field trials cultures of MLN and TI were each done from samples pooled from 3 sites of the particular tissue to limit the numbers of cultures. Care was taken that a full 5 g sample of the pooled tissue was collected for culture. This was
considered a reasonable alternative to 3 separate cultures for each tissue (as done in the pen trial) because in the pen trials most tissue samples were less than 2 g in weight. Of the 44 sheep detected by separate culture of (pooled) MLN, (pooled) TI, ICV and tonsil in the field trials, 42 would have been detected by cultures of (pooled) MLN and (pooled) TI only. One sheep was detected only by tonsil culture and another only from the ICV. Because of the additional time taken to remove tonsils during necropsy, and the minimal extra detection value, tonsil is unlikely to be considered for sampling in further trials. Tonsil would also be highly impractical to sample from lines of sheep at an abattoir, where future monitoring might take place.

The culture of frozen pooled homogenate from the MLN, TI and ICV detected fewer infected sheep than culture of individual tissues, and contamination rates were also higher for the pooled cultures. However, these results are likely to have been influenced by the extra freeze/thaw endured by the pooled samples. Among 19 sheep for which pooled homogenates were cultured alongside the original tissue homogenates, no differences in isolation or contamination rates were seen. A separate study (data not shown) comparing Bactec culture results for freshly prepared and frozen/thawed homogenates demonstrated significant decreases in *M. a. paratuberculosis* isolation rates and significant increases in contamination rates for frozen/thawed samples. Thus culture of freshly pooled homogenate from TI, ICV and MLN may be an economical alternative to culture of individual tissues for the detection of early *M. a. paratuberculosis* infection.

In conclusion, culture of tissues for *M. a. paratuberculosis* successfully detected infected sheep from about 6 to 12 months post-exposure in a range of infected environments. A single pooled tissue culture from each sheep is likely to be an efficient method of examination of groups of sheep. These findings could have practical application in assessing infectivity of pasture, and might reasonably be investigated by abattoir slaughter and sampling of naive sheep of any age which have been running on suspect pastures for 6-12 months.
Chapter 5. Cell mediated immune effector cell populations in early *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep

Summary

Immunoperoxidase labelling (for CD4, CD8, TCR-\(\gamma\delta\), WC1, CD1b, IFN-\(\gamma\), CD45R, CD56, lysozyme and *M. a. paratuberculosis*) was used to investigate changes in cell mediated immune effector cell populations in the intestine and associated lymph nodes in lambs, 2 and 4 months after experimental infection with low doses of *M. a. paratuberculosis*. Although histological lesions were not present, increased numbers of CD4+, TCR-\(\gamma\delta\)+ and WC1+ cells, and fewer CD1b+ cells were demonstrated in infected lambs compared to control lambs. Jejunal Peyer's patches had increased numbers of CD4+, CD8+, TCR-\(\gamma\delta\)+, WC1+ and CD45R+ cells, and decreased numbers of CD56+ fibres compared to ileal Peyer's patches. The significance of these findings in relation to the pathogenesis of ovine Johne's disease is discussed.

Introduction

There has been considerable work on host immunological responses to *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) infection. An enormous body of work examining responses of experimentally infected mice to various mycobacterial diseases of mainly human interest has some relevance to the study of paratuberculosis, and a number of specific studies of *M. a. paratuberculosis* infection in mice have been undertaken. Of the studies undertaken specifically in ruminants most have concentrated on advanced infection in either natural or experimentally induced disease, with particular emphasis on the shift from subclinical to clinical disease. Many studies have used *in vitro* techniques looking at responses of artificially infected cultured macrophages, or stimulated lymphocytes, and other studies have concentrated on systemic rather than local immune responses. While several studies have examined the very earliest stages of infection (the initial uptake of *M. a. paratuberculosis* by the lymphoepithelial tissues of the intestinal PP), there have been few specific studies of the local immunological events occurring in the intestine and regional lymph nodes during the early stages of paratuberculosis infection in ruminants. These local immunological events coinciding with the development of specific adaptive cell mediated immunity (CMI) are likely to be particularly important in determining the outcome of *M. a. paratuberculosis* infection.

Some recent studies in lambs and calves up to 8 weeks post-infection have documented local immunological responses in the absence of demonstrable systemic adaptive immunity. In the sheep studies the dosage of *M. a. paratuberculosis* was \(\geq 10^9\) organisms, and the strain used for experimental infection (originally isolated from deer) had the cultural characteristics of a
bovine strain. Such high doses of *M. a. paratuberculosis* have been shown to usually lead to rapidly progressive disease, unlike natural infection which usually has a prolonged incubation period.\textsuperscript{42} In the present study repeated small doses of an ovine strain of *M. a. paratuberculosis* thought to be more representative of natural infection were given to Australian Merino lambs. The local immunological responses were examined using immunoperoxidase (IPX) labelling of selected cell types during the first 2 to 4 months after initial infection.
Methods

ANIMALS AND EXPERIMENTAL DESIGN:

These were a subset of the weaner lambs used in the pen transmission trial that is described in detail in Chapter 3. The “High dose” lambs (all of which were found to be infected by M. A. paratuberculosis at necropsy) comprised the infected group. None of the lambs had any gross or microscopic lesions consistent with M. A. paratuberculosis infection, although acid-fast bacilli (AFB) were identified in a single macrophage in single lymph node from one infected lamb, and in a foreign-body granuloma in the tonsil of another. All of the infected long-regime lambs and one of the three infected short-regime lambs had positive delayed type hypersensitivity (DTH) to Johnin. Six of the control lambs from the previous study (three each from the short and long regimes) were selected at random to provide the control group in this study. Experimental design is summarised in Table 5.1.

Table 5.1. Experimental design for immunoperoxidase studies.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Freq of Total</th>
<th>Time of necropsy (weeks after</th>
</tr>
</thead>
</table>

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### Table 5.2. Antibodies used for immunoperoxidase labelling.

<table>
<thead>
<tr>
<th></th>
<th>dosing</th>
<th>dose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>first dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>control/short&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 x1 wk</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>control/long&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 x1 wk</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>infected/short</td>
<td>3 x1 wk</td>
<td>1.3x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>infected/long</td>
<td>3 x1 wk</td>
<td>6.9x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>14</td>
</tr>
</tbody>
</table>

<sup>a</sup> short regime (3 doses one week apart, necropsy 5 weeks after last dose)

<sup>b</sup> LONG REGIME (10 DOSES ONE WEEK APART, NECROPSY 5 WEEKS AFTER LAST DOSE)

<sup>c</sup> NUMBER OF VIABLE M. A. PARATUBERCULOSIS DETERMINED BY END-POINT TITRATION IN BACTEC MEDIA.

At necropsy a range of tissues (caecum, colon, ileocaecal valve (ICV), terminal ileum (TI) x 4, jejunum X 2 (with and without a Peyer's patch (PP)), duodenum, mesenteric lymph nodes (MLN) X 4 (ileocaecal node, caudal mesenteric node, two more mesenteric nodes), retropharangeal node and pharangeal tonsil) were collected, snap frozen in liquid nitrogen then stored above liquid nitrogen until processed for immunohistochemistry. These were replicate tissue samples from sites immediately adjacent to those that had been collected into 10% neutral buffered formalin for histopathology in Chapter 3.

In addition several formalin-fixed tissues from adult sheep with typical microscopic lesions of both the multibacillary and paucibacillary forms of Johne's disease were used to evaluate the IPX techniques for lysozyme and M. a. paratuberculosis antigens.

**ANTIBODIES FOR IMMUNOPEROXIDASE LABELLING:**

These are listed in Table 5.2. All are mouse monoclonal antibodies (mAb) raised against ruminant antigens except anti-CD56 (mouse mAb, anti-human), A0099 (rabbit polyclonal antibody, antihuman) and anti-M. a. paratuberculosis (anti-Mptb). Anti-Mptb was an affinity-purified rabbit polyclonal antibody which was prepared locally using a previously described protocol, except that the rabbit was injected subcutaneously with 83 mg of M. a. paratuberculosis (strain 316V) in two doses one month apart. The antibody preparation was stored as a 1:10 solution in 25mM Tris-HCl, pH 7.4, 0.15 M NaCl and 50% glycerol at -20 °C, and diluted in PBS, pH 7.4 to 1:500 immediately before use. The specificity when used in IPX has not been determined.
<table>
<thead>
<tr>
<th>Antibody (Clone)</th>
<th>Working dilution</th>
<th>Specificity</th>
<th>Cell type</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBU-T4 (44.38+44.97)</td>
<td>1:4</td>
<td>CD4</td>
<td>αβ T helper lymphocytes</td>
<td>Centre for Animal Biotechnology, University of Melbourne</td>
<td>199</td>
</tr>
<tr>
<td>SBU-T8 (24.96)</td>
<td>1:4</td>
<td>CD8</td>
<td>αβ T lymphocytes (cytotoxic)</td>
<td>Centre for Animal Biotechnology, University of Melbourne</td>
<td>199</td>
</tr>
<tr>
<td>Anti-γδ (86D)</td>
<td>undiluted</td>
<td>TCR-γδ</td>
<td>γδ T lymphocytes</td>
<td>Basel Institute for Immunology</td>
<td>198</td>
</tr>
<tr>
<td>CC15</td>
<td>undiluted</td>
<td>WC1 (T19)</td>
<td>γδ T lymphocytes</td>
<td>AFRC Compton</td>
<td>142, 198</td>
</tr>
<tr>
<td>CC20</td>
<td>undiluted</td>
<td>CD1b</td>
<td>various, macrophages, dendritic cells nerve fibres</td>
<td>AFRC Compton</td>
<td>247</td>
</tr>
<tr>
<td>Anti-CD56</td>
<td>1:50</td>
<td>CD56</td>
<td></td>
<td>Becton Dickenson</td>
<td>315</td>
</tr>
<tr>
<td>Anti-CD45R (73B)</td>
<td>undiluted</td>
<td>CD45R</td>
<td>B lymphocytes and some activated T lymphocytes</td>
<td>Basel Institute for Immunology</td>
<td>201, 200</td>
</tr>
<tr>
<td>IFN-9</td>
<td>undiluted</td>
<td>IFN-γ</td>
<td>T lymphocytes, possibly extracellular</td>
<td>CSIRO Livestock Industries</td>
<td>278</td>
</tr>
<tr>
<td>A0099</td>
<td>1:100</td>
<td>lysozyme</td>
<td>activated macrophages, paneth cells</td>
<td>Dako</td>
<td>255</td>
</tr>
<tr>
<td>anti-Mptb</td>
<td>1:500</td>
<td>M. a. paratb?</td>
<td>infected macrophages</td>
<td>EMAI, NSW Agriculture</td>
<td></td>
</tr>
</tbody>
</table>

**IMMUNOPEROXIDASE LABELLING:**

CD4, CD8, TCR-γδ, WC1, CD1b, IFN-γ. 8μm sections were cut from the frozen tissues on a cryostat and mounted on glass slides. Slides were foil-wrapped and stored at -80 °C until stained. All subsequent procedures were performed at room temperature unless otherwise stated. Slides were thawed for one hour, unwrapped, fixed for 5 min in formal calcium (23 mL 35% formalin, 2 g CaCl₂, and distilled H₂O to 200 mL, pH to 7.2–7.4 with dilute NaOH at 4 °C) at 4 °C, then rinsed in distilled H₂O. Slides were then further fixed and endogenous peroxidase removed using a 5 min incubation in methanol peroxide (6% by volume of 30% hydrogen peroxide in methanol prepared immediately before use), then washed twice in phosphate buffered saline (PBS). Slides were then blocked for 20 min in PBS containing 0.5% bovine serum albumin (BSA) and 1% sheep serum. Slides were not washed prior to incubation for one hour with the selected primary antibody. After washing in PBS, slides were incubated with the secondary antibody (biotinylated sheep antimouse, 1:500 in 0.25% BSA in PBS, Sigma Immunochemicals) for one hour,
washed in PBS, incubated with Streptavidin-horseradish peroxidase complex (Vector Laboratories) for 30min, then washed in PBS. Slides were then incubated with the chromagen solution (6 mg diaminobenzidine (DAB) in 10mL 0.05 M Tris buffer, pH 7.6, and 10μL 30% H₂O₂ added immediately prior to use), and monitored until sufficient colour development had occurred (usually about 2 min). After a final rinse in tap water slides were counterstained lightly with Harris haematoxylin, dehydrated through graded alcohols and mounted in DePex mounting medium (BDH). Negative control slides in which the primary antibody was replaced by PBS were processed concurrently for each section.

**CD56, CD45R:** This was performed exactly as above up to and including incubation with primary antibody except that the blocking solution contained 1% goat serum in place of sheep serum. The secondary antibody (Envision +, goat anti-mouse, bound to dextran and horseradish peroxidase, Dako) was incubated for 30 min at 37 °C. Following washing in PBS slides were incubated for 5 min in the chromagen solution (0.002 g aminoethylcarbazole (AEC) dissolved in 200μL dimethylformamide, added to 10 ml acetate buffer, pH 5, containing 5 μL Tween-20, and 5 μL of 30% H₂O₂ added immediately prior to use). Slides were then washed in distilled water, counterstained lightly in haematoxylin, washed in distilled water and mounted in Faramount (Dako).

**Lysozyme, Mptb:** For cryostat sections this was performed exactly as described for CD56 and CD45R except that the primary antibody was incubated for 90 min and the secondary antibody was Envision +, goat anti-rabbit (Dako). For formalin-fixed material tissues were fixed overnight in 10% buffered neutral formalin and routinely embedded in paraffin blocks for histopathology. 5μm sections were cut and mounted on poly-L-lysine coated slides and dried at 60 °C for 20 min. Sections were dewaxed through graded alcohols to water, then incubated for 30 min in 0.2% trypsin in 0.1% CaCl₂ at 37 °C to unmask antigens. Following a rinse in distilled water, the slides were placed in methanol peroxide and this and subsequent steps were exactly as for the cryostat sections.

**Microscopic examination:**

All sections were examined using bright-field microscopy. Haematoxylin and eosin (H&E) stained sections were examined routinely for lesions, scanning first under lower powers, then focusing on suspicious areas under higher magnification up to 400 X. For Ziehl-Neelsen (ZN)
stained sections particular attention was focused on any suspicious areas identified in H&E sections and also on the intestinal PP and the paracortical and subcapsular areas of the MLN. These were examined thoroughly under 400 X magnification and any fields containing material suspicious of being AFB were examined under oil-immersion. A similar procedure was followed for sections labelled for *M. a. paratuberculosis* antigens.

For each of the other antigens, IPX labelled sections were examined initially at lower powers (40 to 100 X magnification) to provide a subjective assessment of the general distribution and abundance of labelled cells in each tissue (irrespective of infection status or time). A brief description of this subjective assessment for intestinal, MLN and tonsillar sections is given at the beginning of the results for each antigen, and illustrated for TI and MLN by a typical example of each. In the colon and caecal sections of many sheep and the tonsil and ICV sections of several sheep, the lymphoid tissue was not present on the prepared slides, and examination of these tissues was limited to subjective assessment. More detailed examination was undertaken of the TI, jejunal and MLN sections, and the numbers of positively stained cells within particular micro-anatomical regions (sub-tissues) were counted or estimated to provide data for statistical analysis. For intestinal sections the sub-tissues comprised the mucosa (villi and crypts), the domes of the PP, the interfollicular areas, and finally the follicles. In the MLN sub-tissues were the follicles, the paracortex and the medulla. For each antigen one or more undeformed and clearly stained, non-overlapping fields covering each of the sub-tissues from each of up to three different sections of TI and from a single section of jejunum (including PP) were assessed from each sheep. Similarly, fields covering each sub-tissue from sections of at least 3 different MLN per sheep were examined. The number of fields available for reliable assessment on each section varied between sections. Not all sub-tissues were included in the analysis for each antigen, for example when few or no labelled cells were present in a particular sub-tissue, or when so many were present that differential assessment was not possible. The specific counting techniques were varied to suit the different morphology, numbers and distribution of labelled cells for the different antigens as follows.

For analysis of CD4, CD8, CD56 and sub-tissues for CD 45R except intestinal mucosa, a semi-quantitative five-point scoring system was devised. For each sub-tissue, labelled cells or fibres were counted or estimated in as many assessable “equivalent 200 X” fields as were present on each section. An “equivalent” field assumes that the sub-tissue occupied all of the visible field. Fields containing less than 25% of the relevant sub-tissue were not used. For example if the
sub-tissue of interest occupied only 50% of a particular field the number of labelled cells counted or estimated for that sub-tissue in that field would be multiplied by 2 to give the number in an “equivalent” field. For CD4 and CD8, fields were scored as follows: 0 (0-1 labelled cells), 1 (2-10 cells), 2 (11-50 cells), 3 (51-250 cells) and 4 (>250 cells). For CD45R, scores were assigned as follows: 1 (4-10 cells labelled), 2 (11-30), 3 (31-100), 4 (101-300) and 5 (>300). For CD56 the score was based on the subjective appearance of the labelled fibres (counting was not possible) as follows: 0 (no fibres labelled), 1 (only occasional fragments of fibres), 2 (diffusely scattered fibre fragments), 3 (fibre fragments often coalescing) and 4 (mostly continuous, confluent fibres). For all the above, the median score for the fields assessed on a section was taken as the best estimate of the sub-tissue score for that section. The mean of the scores for each section from a particular sheep was the sub-tissue value used for analysis.

For analysis of TCR-γδ and WC1, most sub-tissues had numbers of labelled cells that facilitated accurate counting, and the subjective distribution of labelled cells within particular sub-tissues was fairly uniform. All labelled cells within typical 400 X fields containing mostly the sub-tissue of interest were counted. In the TI and MLN, three fields (one from each of three different sections) were counted for each sub-tissue for each sheep, while in the jejunum two fields for each sub-tissue were counted from a single section. A similar approach was used to count CD45R-labelled cells in the intestinal mucosa. In this case, four 400 X fields of mucosa from one section each of TI and jejunum were made for each sheep. The mean of the counts for each sub-tissue from a particular sheep was the value used for analysis.

The distribution of CD1 labelled cells required a different counting system. Labelled cells were concentrated in the interfollicular areas of the PP and along the mucosa/submucosa border at the level of the muscularis mucosae. For statistical analysis of interfollicular areas of intestinal sections, all labelled cells in up to five 400 X fields per section were counted. In the jejunal sections the fields usually contained only interfollicular tissue. In the ileum, fields (avoiding obvious domes) were centred on the muscularis mucosae and all labelled cells counted. These were mainly interfollicular cells but included also some in the lamina propria around the bases of the crypts. For analysis of domes, all labelled cells within each of up to five well sectioned domes per section (including epithelium and lamina propria) were counted under 400 X magnification. For the ileum whole domes always fitted within 400 X fields. In the jejunum domes were sometimes larger than a 400 X field, and in that case only labelled cells within the
particular field (centred on the dome with the epithelium at one edge of the field) were counted. For each sub-tissue in the MLN all labelled cells in up to three 400 X fields per section from three different nodes were counted. The means for each sheep of the counts for each sub-tissue formed the data for analysis (mean of up to 15 fields for terminal ileal interfollicular areas, up to five fields for other intestinal sub-tissues, and up to nine fields for each of the MLN sub-tissues).

Statistical analysis:
For each antigen and each sub-tissue, the means of multiple counts or scores from each sheep were the data used for analysis with Minitab statistical software. A 2-way analysis of variance (ANOVA) (infection-status and time) was used wherever possible, and unless otherwise specified in the results section, analysis was by ANOVA. For data sets that were not normally distributed (Anderson-Darling Normality Test), for which variances were not homogeneous (Bartlett’s test), or where there were missing values (for example if PP was not present in intestinal sections), the non-parametric Kruskal-Wallis (KW) test was used was used to compare all infected with all control sheep, and all long-regime with all short-regime sheep. Student’s paired t test was used for comparisons between TI and jejunal sub-tissues labelled for the same antigen, and to compare the results for TCR-γδ and WC19.

Results

CD4:
In the intestinal mucosa moderate numbers of labelled cells were present in the lamina propria between crypts and within the domes of the PP. Moderate to large numbers of labelled cells were seen in the lamina propria of the villi but no intraepithelial cells were labelled. Few cells were labelled within the follicles of the PP (these were excluded from the statistical analysis) and moderate to large concentrations were present in the interstitial areas. In the MLN there were moderate numbers of labelled cells within the follicles and medullary regions. In the paracortical areas there too many labelled cells to count, so this sub-tissue was excluded from statistical analysis. In the tonsil there were very large numbers of CD4+ cells in the interfollicular zones and moderate numbers within the epithelium, lamina propria and also in the follicles. The general distribution and abundance of labelled cells is illustrated in Figures 5.1a and b, and the mean results for the groups of sheep are given in Table 5.3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal ileal PP</th>
<th>Jejunal PP</th>
<th>Mesenteric lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Data for jejunal sub-tissues were not normally distributed and the KW test was used. The number of CD4 positive cells was greater in the infected than control sheep in each sub-tissue.
of the TI - mucosa, domes (P<0.05), and interfollicular (P<0.01). Similarly, in all jejunal sub-tissues the infected sheep had higher numbers of positively stained cells than the control sheep, but this was only significant for the interfollicular areas (P<0.05). There were no significant differences with regard to time in the intestine, although a trend was apparent in that the means for all sub-tissues of the long-regime sheep were higher than those of the short-regime sheep. No infection-time interaction was detected. In the MLN no significant differences with regard to infection or time were detected, although a similar trend for infected sheep to have increased the numbers of CD4$^{+}$ cells was apparent. The interfollicular area of the jejunal PP had significantly more CD4$^{+}$ cells than that of the terminal ileum (P <0.01) and a similar trend was apparent for the dome area (P=0.1). There was no difference in the mucosa.

**CD8:**

Within the intestinal mucosa labelled cells were abundant both in the villous lamina propria and epithelium and present in moderate numbers in the lamina propria and epithelium of the crypts and domes. Moderate numbers were present in the interfollicular areas of the PP but few cells were labelled in the follicles (these were excluded from the statistical analysis). In the MLN very large numbers of cells were labelled in the paracortex and moderate to large numbers in the medulla. Smaller numbers of labelled cells were present in the follicles. In the tonsil there were large numbers of CD8$^{+}$ cells in the interfollicular areas, moderate numbers in the epithelium and lamina propria but few positive cells within the follicles. The general distribution and abundance of labelled cells is illustrated in Figures 5.2a and b, and the mean group results are given in Table 5.4.

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal ileal PP</th>
<th>Jejunal PP</th>
<th>Mesenteric lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mucosa</td>
<td>domes</td>
<td>interfollicular</td>
</tr>
<tr>
<td>Control/short</td>
<td>2.833</td>
<td>2.250</td>
<td>2.111</td>
</tr>
<tr>
<td>Control/long</td>
<td>2.750</td>
<td>1.833</td>
<td>2.500</td>
</tr>
<tr>
<td>Infected/short</td>
<td>2.694</td>
<td>2.111</td>
<td>2.667</td>
</tr>
<tr>
<td>Infected/long</td>
<td>2.722</td>
<td>2.250</td>
<td>2.667</td>
</tr>
</tbody>
</table>

Table 5.4. CD8 analysis. Means of scores from 3 sheep in each group.
Data for sub-tissues other than mucosa and domes from TI and for medulla and follicles of the MLN were not normally distributed and the KW test was used. No significant differences were detected with regard to infection status or time, nor was there any significant infection-time interaction. In the MLN there was a trend in the paracortex and medulla for short-regime sheep to have higher numbers of CD8 positive cells (P<0.1). The interfollicular area of the
jejunal PP had significantly more CD8\(^+\) cells than that of the TI (P = 0.001), but there were no differences in the mucosa or domes.

**TCR-\(\gamma\delta\):**

In the intestinal sections there were few or no positive cells in follicles of the PP (excluded from statistical analysis) but moderate to large numbers in interfollicular areas, and both epithelium and lamina propria of the domes, villi and crypts. In the MLN moderate to large numbers of positive cells were present in the medulla and paracortex and smaller numbers in the follicles. There were moderate to large numbers of labelled cells in the epithelium, lamina propria and interfollicular areas of the tonsil, but few or no positive cells in the follicles. The general distribution and abundance of labelled cells is illustrated in Figures 3a and b, and mean group results are given in Table 5.5.

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal ileal PP</th>
<th>Jejunal PP</th>
<th>Mesenteric lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mucosa</td>
<td>domes</td>
<td>interfollicular</td>
</tr>
<tr>
<td>Control/short</td>
<td>66.7</td>
<td>16.0</td>
<td>32.3</td>
</tr>
<tr>
<td>Control/long</td>
<td>85.5</td>
<td>19.7</td>
<td>26.7</td>
</tr>
<tr>
<td>Infected/short</td>
<td>81.8</td>
<td>21.0</td>
<td>42.3</td>
</tr>
<tr>
<td>Infected/long</td>
<td>92.7</td>
<td>33.3</td>
<td>47.7</td>
</tr>
</tbody>
</table>

[Figure 5.3a. TCR-\(\gamma\delta\) labelled cells in terminal ileum, x 100](image)
Data for the domes of the TI, interfollicular areas of the jejunum and follicles and medulla of the MLN were not normally distributed and the KW test was used. Infected lambs had significantly more cells labelled in jejunal mucosa and interfollicular areas (P<0.05) than control lambs. A similar trend was seen in the domes of the jejunal PP and all sub-tissues of the TI. In the ileal mucosa long-regime lambs had more γδ+ cells than the short-regime lambs (P<0.05) but there was no similar trend in other sub-tissues. There were no significant changes in the MLN sub-tissues associated with infection or time. No infection-time interactions were detected. The domes of the jejunal PP had significantly more cells labelled for TCR-γδ than those of the terminal ileum (P =0.001). A similar trend was apparent for the interfollicular area (P<0.1), but there was no difference in the mucosa.

WC1:

Subjectively the numbers and distributions of the labelled cells were similar to TCR-γδ but there appeared to be fewer cells labelled in the epithelium of intestinal sections. Figures 5.4a and b illustrate the general distribution and abundance of labelled cells, and mean group results are given in Table 5.6.

Table 5.6. WC1 analysis. Means of counts from 3 sheep in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal ileal PP</th>
<th>Jejunal PP</th>
<th>Mesenteric lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mucosa</td>
<td>domes</td>
<td>interfollicular</td>
</tr>
</tbody>
</table>

142
<table>
<thead>
<tr>
<th></th>
<th>WC1</th>
<th>19.595</th>
<th>19.074</th>
<th>52.000</th>
<th>35.722</th>
<th>43.833</th>
<th>5.963</th>
<th>62.185</th>
<th>37.667</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/short</td>
<td>36.840</td>
<td>14.766</td>
<td>21.333</td>
<td>51.000</td>
<td>38.667</td>
<td>66.667</td>
<td>10.296</td>
<td>66.000</td>
<td>47.333</td>
</tr>
<tr>
<td>Control/long</td>
<td>39.907</td>
<td>18.917</td>
<td>33.259</td>
<td>65.333</td>
<td>38.833</td>
<td>59.778</td>
<td>10.481</td>
<td>80.370</td>
<td>82.926</td>
</tr>
<tr>
<td>Infected/short</td>
<td>51.704</td>
<td>38.889</td>
<td>45.000</td>
<td>67.611</td>
<td>55.111</td>
<td>75.333</td>
<td>12.963</td>
<td>84.037</td>
<td>60.519</td>
</tr>
<tr>
<td>Infected/long</td>
<td>58.241</td>
<td>38.889</td>
<td>51.000</td>
<td>65.333</td>
<td>38.833</td>
<td>59.778</td>
<td>10.481</td>
<td>80.370</td>
<td>82.926</td>
</tr>
</tbody>
</table>

Figure 5.4a. WC1 labelled cells in terminal ileum, x 100

Figure 5.4b. WC1 labelled cells in mesenteric lymph node, x 100
Data for interfollicular areas and domes of the TI and for MLN follicles were not normally distributed, and there were some missing values for the jejunal data so the KW test was used. In the mucosa (P<0.001) and interfollicular areas (P<0.05) of the TI there were significantly more labelled cells in the infected lambs than the controls. The same trend was apparent in the other intestinal sub-tissues and the differences for TI domes and jejunal mucosa approached significance (P<0.1). In the MLN the same trend for more WC1+ cells in the infected lambs was seen in all sub-tissues, and approached significance for medulla (P<0.1) and paracortex (P=0.1). With regard to time, in every sub-tissue examined there were more WC1+ cells in the long-regime lambs than the short-regime lambs, although individual test results were not statistically significant. No infection-time interactions were detected. In each sub-tissue of the jejunal PP there were significantly more cells labelled for WC1 than in the corresponding sub-tissue of the TI (P <0.01). A comparison was also made between cells labelled for TCR-γδ and WC1. Significantly more cells were labelled for TCR-γδ than for WC1 in the mucosa of both the TI (P<0.001) and jejunum (P<0.01), in the interfollicular area of the TI (P<0.01), and in the follicles (P<0.01) and medulla (P<0.05) of the MLN.

CD1b:

There were moderate to large numbers of labelled cells in the interfollicular areas of the intestinal PP, and in the lamina propria of the domes. Smaller numbers were seen in the dome epithelium and in the lamina propria surrounding the intestinal crypts, but labelled cells were rarely present in the intestinal epithelium, in the lamina propria of the villi or in the follicles of the PP. In the MLN there were large numbers of CD1b+ cells in the paracortex and small numbers in the medulla, but few or none within the follicles. In the tonsil positive cells were frequent in the interfollicular areas and in smaller numbers in the epithelium and lamina propria, with few or none in follicles. In the paracortical areas of the MLN and to a lesser extent in the interfollicular areas of the intestinal PP and tonsil the distribution of CD1b+ cells was noticeably irregular with locally extensive areas having large numbers of cells and other areas with few or moderate numbers. In the TI the CD1b+ cells in the interfollicular areas were concentrated directly beneath the muscularis mucosae, with very few cells labelled deeper between the follicles. Figures 5.5a and b illustrate the general distribution and abundance of labelled cells, and mean group results are given in Table 5.7.

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal ileal PP</th>
<th>Jejunal PP</th>
<th>Mesenteric lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>domes</td>
<td>interfollicular</td>
<td>domes</td>
</tr>
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Table 5.7. CD1b analysis. Means of counts from 3 sheep in each group
<table>
<thead>
<tr>
<th></th>
<th>Control/short</th>
<th>Control/long</th>
<th>Infected/short</th>
<th>Infected/long</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18.603</td>
<td>21.787</td>
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<td>34.817</td>
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<td>18.000</td>
</tr>
<tr>
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<td>40.600</td>
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</tr>
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<td>2.067</td>
<td>1.233</td>
<td>1.800</td>
<td>1.300</td>
</tr>
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<td></td>
<td>8.333</td>
<td>8.933</td>
<td>6.833</td>
<td>4.767</td>
</tr>
</tbody>
</table>

**Figure 5.5a.** CD1b labelled cells in terminal ileum, x 100

**Figure 5.5b.** CD1b labelled cells in mesenteric lymph node, x 100
There were some missing values for the jejunal data and the KW test was used. In the intestine there were no significant differences with regard to infection status or time for any sub-tissue, nor any infection-time interaction. In the MLN there were significantly fewer cells expressing CD1b in the paracortical areas of the infected sheep compared to the control sheep (P<0.01) and also a significant time-infection interaction (P<0.05) with long-regime infected sheep showing a greater reduction in CD1b+ cells than short-regime infected sheep. No differences in numbers of CD1b+ cells were detected between corresponding sub-tissues of the terminal ileum and jejunum.

**IFN-γ:**

Very few cells were labelled in any tissue and statistical analysis of the data was not possible. In most lambs there were very rare positive cells in the lamina propria surrounding the crypts in one or a few intestinal sections. Some sheep also had very occasional positive cells in the interfollicular areas, domes and follicles of the PP. Positive cells were never seen in the mucosal epithelium. Several sheep had occasional positive cells in the interfollicular areas or epithelium of the tonsil. Two sheep (both from the infected/short group) had single follicles in the ileal PP with >10 labelled cells, and a single sheep from the infected/long group had similar findings in one tonsillar follicle. In the MLN there were occasional positively labelled cells in paracortex, medulla or follicles of several sheep. One sheep from the control/long group had a single follicle in the MLN with >10 labelled cells.

**CD56:**

Nerve fibres rather than cells are labelled by antibody to CD56. Labelled fibres were abundant in the lamina propria of the intestinal mucosa. These were often aligned along the epithelial basement membrane of the crypts but this effect was less noticeable in the villi. In the PP, labelled fibres strikingly outlined the margins of the follicles but only small numbers of fibres were apparent in the interfollicular areas or in the domes, and few or none were present within follicles. In the MLN, labelled fibres were far less prominent than in the intestine, with small numbers sometimes outlining follicular margins, and scattered fibres, usually associated with blood vessels, present in the paracortex and medulla. There were too few labelled fibres in the MLN for meaningful analysis. Figures 5.6a and b illustrate the general distribution and abundance of labelled cells, and mean group results are given in Table 5.8.

<table>
<thead>
<tr>
<th>Table 5.8. CD56 analysis. Means of scores from 3 sheep in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control/short</td>
</tr>
</tbody>
</table>

146
<table>
<thead>
<tr>
<th></th>
<th>1.861</th>
<th>2.000</th>
<th>0.722</th>
<th>4.000</th>
<th>1.667</th>
<th>2.000</th>
<th>0.750</th>
<th>3.000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/long</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected/short</td>
<td>1.778</td>
<td>2.000</td>
<td>0.972</td>
<td>3.889</td>
<td>1.750</td>
<td>1.667</td>
<td>1.333</td>
<td>3.000</td>
</tr>
<tr>
<td>Infected/long</td>
<td>1.944</td>
<td>2.000</td>
<td>0.278</td>
<td>3.889</td>
<td>1.833</td>
<td>2.000</td>
<td>0.333</td>
<td>2.667</td>
</tr>
</tbody>
</table>

Figure 5.6a. CD56 labelled nerve fibres in terminal ileum, x 100

![Image of CD56 labelled nerve fibres in terminal ileum](image1)

Figure 5.6b. CD56 labelled nerve fibres in mesenteric lymph node, x 120

![Image of CD56 labelled nerve fibres in mesenteric lymph node](image2)

Except for the mucosa and domes of the TI the data were not normally distributed and the KW test was used. No significant differences between groups were detected with regard to
infection-status or time. There were significantly fewer CD56$^+$ fibres in the follicular margins of the jejunal PP than in those of the ileum (P<0.001).

**CD45R:**

There were moderate to large numbers of distinctly labelled cells in the intestinal mucosal sections. In the interfollicular areas of the PP there were very large numbers of labelled cells and in the follicles it was not possible to determine at all which cells were labelled. The whole follicle was lightly, but diffusely, labelled so follicles were omitted from statistical analysis. In the MLN large to very large numbers of cells were labelled in the paracortex and medulla, and the follicles were similar to those of the PP. In the tonsils large to very large numbers of CD45R$^+$ cells were present in the epithelium and lamina propria, and the follicles were similar to those of the PP. Figures 5.7a and b illustrate the general distribution and abundance of labelled cells, and mean group results are given in Table 5.9.

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal ileal PP</th>
<th>Jejunal PP</th>
<th>Mesenteric lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mucosa$^a$</td>
<td>domes</td>
<td>interfollicular</td>
</tr>
<tr>
<td>Control/short</td>
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<td>4.011</td>
<td>3.889</td>
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<td>Control/long</td>
<td>119.1</td>
<td>3.500</td>
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<tr>
<td>Infected/short</td>
<td>116.3</td>
<td>4.167</td>
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<tr>
<td>Infected/long</td>
<td>116.8</td>
<td>3.778</td>
<td>4.111</td>
</tr>
</tbody>
</table>

Figure 5.7a. CD45R labelled cells in terminal ileum, x 100
Data for the TI interfollicular area was not normally distributed and there were some missing values in the jejunal data and the mucosa of the TI, so the KW test was used. No significant differences between groups were detected with regard to infection-status or time. Each sub-tissue of the jejunum had more CD45R⁺ cells than the corresponding TI sub-tissue (mucosa (P<0.05), domes (P<0.01), interfollicular (P<0.1)).

LYSOZYME:

In formalin-fixed tissues from adult sheep with microscopic lesions of Johne's disease, sections labelled for lysozyme demonstrated moderate labelling of clumps of macrophages. These were easier to visualise than in the H&E stained sections. Paneth cells in the crypts of the intestine were also intensely labelled and there was minimal non-specific or background staining. See Figures 5.8a-c. In the cryostat sections, however, the Paneth cells were not consistently labelled, and some diffuse background staining was present, most prominent in the medullary sinuses of the MLN. Also, the removal of endogenous peroxidase was not complete in the cryostat sections, with scattered cells (probably eosinophils) and some scattered debris strongly labelled in both the negative control and test slides. Several sheep had scattered cells labelled (in the test slides only) with a granular appearance in the domes of the intestinal PP, the interfollicular areas or the lamina propria. In the intestinal PP and MLN several sheep showed non-granular labelling, almost hyaline in appearance, of large macrophages within the centres of the follicles.
There was no apparent association of labelling with either infection status or time and the results were too few and inconsistent for any meaningful analysis. In one sheep from the infected/long group a clump of macrophages was labelled in the interfollicular area of the tonsil. This clump was not present on the routine H&E or ZN stained sections from an adjacent piece of tonsil. Further cryostat sections cut from the same piece of tissue as the lysozyme section were stained with ZN or labelled for *M. a. paratuberculosis* (see below). In both these sections there was evidence for a piece of vegetable foreign matter within the lesion. A single AFB was present in a macrophage at the edge of the lesion in the ZN stained section, and a single labelled organism was also seen in the *Mptb*-labelled section in a different part of the lesion.

Figure 5.8a. Clumps of epithelioid macrophages in lamina propria of terminal ileum from a sheep with paucibacillary Johne’s disease, haematoxylin and eosin, x 400
MPTB:

In formalin-fixed tissues from adult sheep with multibacillary lesions of Johne's disease, labelled organisms were visible in IPX sections in about the same numbers as were visible as AFB in ZN stained sections. In some paucibacillary cases more organisms were seen in the IPX sections, but this was not a consistent finding. See Figures 5.9a and b. In many of the cryostat sections failure of complete removal of endogenous peroxide as in the lysozyme slides
precluded the confident identification of small numbers of truly labelled antigens. Certainly no large numbers of labelled antigens were seen in any tissue of the lambs from the infection trial. Fortuitously, in the tonsil section of the sheep with a macrophage clump, the control section was clear of non-specific staining in the interstitial area.

Figure 5.9a. Clumps of epithelioid macrophages in lamina propria of terminal ileum from a sheep with paucibacillary Johne’s disease, Ziehl-Neelsen, x 400

Figure 5.9b. Clumps of epithelioid macrophages in lamina propria of terminal ileum from a sheep with paucibacillary Johne’s disease, labelled for \textit{M. a paratuberculosis}, x 400

\textit{M. a. paratuberculosis} antigens are clearly labelled

Acid-fast bacilli are difficult to locate
Discussion

Little work has been done on the immunological events in the early stages of *M. a. paratuberculosis* infection. In the only recent studies conducted in sheep, relatively large doses of *M. a. paratuberculosis* of non-ovine origin were used.\textsuperscript{24,26} The present study, using lower doses of ovine stains of *M. a. paratuberculosis* may be more representative of the changes occurring during natural infection. In this study, no histological lesions were present in the intestine or MLN, including in sections labelled for lysozyme which greatly facilitates the detection of clumps of activated macrophages.\textsuperscript{255} *M. a. paratuberculosis* was, however, recovered from at least one tissue of every infected lamb, demonstrating successful infection. Intestinal lesions have been reported from other studies in both sheep and cattle in the first few months after experimental infection with bovine strains, usually at much higher doses.\textsuperscript{26,150,241,250} Such lesions consist of accumulations of epithelioid cells closely associated with, and sometimes within, the follicles of the PP. The lack of lesions in this study may be due to the use of lower infectious doses, or possibly a strain effect, and is consistent with results of field trials (Chapter 4) in which lesions were never detected in naturally exposed sheep after exposure periods of less than 8 months.

The timing of necropsy in the present study was mainly aimed at maximising the numbers of sheep which might be positive for *M. a. paratuberculosis* by culture of tissues at necropsy (Chapter 3), and coincides roughly with the development of measurable systemic CMI. These lambs were just beginning to develop DTH reactions with one of three infected lambs positive at 6 weeks post-infection, and three of three infected lambs positive at 13 weeks post-infection. Other studies using IFN-γ assay have first detected systemic CMI 9-18 weeks after infection.\textsuperscript{130,314,318} Specific local CMI responses may develop earlier. In experimentally infected calves increased IFN-γ production by stimulated caecal MLN cells was detected 6 weeks post-infection,\textsuperscript{58} whereas in lambs specific local CMI responses to mycobacterial antigens were not observed up to 6 weeks after infection.\textsuperscript{26} Examination of lambs at two distinct time periods after infection in the current study allowed some investigation into effects of increasing age on the immunological parameters studied and a possible insight into changes as adaptive immunity develops.

An overview of the results from the current study shows that infection with *M. a. paratuberculosis* was associated with increased numbers of CD4\textsuperscript{+} and γδ\textsuperscript{+} cells in most sub-tissues of the TI and jejunal PP as well as in the MLN. Infection was also associated with a decrease in the numbers of cells expressing CD1b in the MLN. Comparing the two time periods (ie sheep of about 5
months to sheep 7 months old) indicated that numbers of CD4+ and γδ+ cells in intestine and MLN tended to increase with age, while numbers of CD8+ cells in the MLN tended to decrease with age. This study also confirmed differences between the ileal and jejunal PP of the young ruminant. Sub-tissues of the ileal PP had lower levels of CD4+, CD8+, γδ+ and CD45R+ than corresponding sub-tissues of the jejunum.

It is generally agreed that protection against mycobacterial disease is mainly due to CD4+ Th1 cells that produce IFN-γ to activate macrophages which then kill mycobacteria during phagocytosis.298 In this study, increased numbers of CD4+ cells were indeed seen in all intestinal sub-tissues of infected sheep, and in the medulla and follicles of the MLN. This is consistent with previous observations in lambs given significantly higher doses of bovine strain and examined up to 6 weeks post infection,26 and with the notion of a developing early cell mediated response. IPX for IFN-γ was attempted in this study but the results were too inconsistent to indicate any effect of infection in increasing the detectable levels in local tissues. An increase in in vitro IFN-γ production by M. a. paratuberculosis-stimulated peripheral blood leukocytes was demonstrated in the infected group of sheep (Chapter 3), but measurement of local stimulated IFN-γ production was not undertaken in this study. Previous studies in sheep up to 6 weeks post-infection failed to detect any increase in IFN-γ production by intestinal or MLN lymphocytes after incubation with mycobacterial antigens.26

Several studies indicate a protective role for γδ T cells in mycobacterial infections such as tuberculosis and leprosy in man.18 224 Studies with γδ-knockout mice suggest that γδ T cells play an important role by promoting the influx of lymphocytes and monocytes, and limiting the access of inflammatory cells that do not contribute to protection, but do not themselves directly contribute to protection against tuberculosis.82 Similar studies failed to demonstrate a protective role for these cells in experimental M. a. paratuberculosis infections in mice but did suggest a crucial role in the development of epithelioid granulomas.331 The situation in ruminants may be quite different. Young ruminants have high numbers of γδ T cells in the circulation and the intestinal mucosa, suggesting a particular role for these cells in early immune protection at mucosal surfaces.248(p517) However, most specific ruminant studies have failed to demonstrate any protective effect of γδ T cells in paratuberculosis. Studies in subclinically infected cattle have shown that CD4+ T cells and to a lesser extent CD8+ T cells produce IFN-γ in response to M. a. paratuberculosis, but none is produced by γδ T cells.19 High numbers of circulating γδ T cells have been associated with disseminated lesions of paratuberculosis in goats318 and with depression of CD4+ cell proliferation in cattle.61 IPX studies demonstrated an increased relative percentage of γδ
T cells in the ileum of sheep with advanced paratuberculosis. Only a few studies have investigated a role for $\gamma\delta$ T cells in early *M. a. paratuberculosis* infection. A recent *in vitro* study indicated that after 5 days stimulation with *M. a. paratuberculosis* antigens the levels of IL-2R expression by $\gamma\delta$ T cells from uninfected goats approached that from infected goats, possibly indicating activation due to a first line of defence against mycobacterial antigens. And two Scottish studies demonstrated increased numbers of $\gamma\delta$ T cells in intestinal PP or MLN of lambs in the first weeks post-infection. This study reinforces those recent observations that $\gamma\delta$ T cells are involved in early *M. a. paratuberculosis* infection of sheep, but does not indicate whether such response may be beneficial or otherwise. Two different antibodies were used in this study to label $\gamma\delta$ T cells, CC15 and 86D. CC15 labels WC1 (T19), a surface antigen expressed on a subset of $\gamma\delta$ T cells. A large proportion of $\gamma\delta$ T cells in intestinal epithelium lack T19. 86D labels cells with TCR-$\gamma\delta$, and these also include a small percentage of CD8+ cells. The findings in this study are consistent with these reports. Antibody 86D labelled more cells than CC15 in the mucosa of the intestine, and fewer intraepithelial cells were labelled with CC15. Despite differences in the specificity of the two antibodies similar conclusions were reached for each, with increased numbers of $\gamma\delta$ T cells in the intestinal tissues of the infected lambs, and also a trend for increasing numbers of labelled cells with increasing age.

The CD1 system may be important in the innate immune response via antigen independent recognition and lysis of cells expressing CD1 by $\gamma\delta$ T cells and NK-T cells, providing a rapid response in the early phases of mycobacterial infection. CD1 molecules are also involved in the presentation of lipid and glycolipid antigens (including mycobacterial LAM) to T cells leading to specific adaptive immune responses. In humans the expression of CD1 correlates directly with effective immunity to *M. leprae*, and there is *in vitro* evidence for down-regulation of CD1 expression on APC’s in virulent *M. tuberculosis* infection. In the only reported study to date of CD1 involvement in paratuberculosis no changes in CD1 expression were found in PP and MLN of lambs one month after infection. In this study there were significantly fewer cells expressing CD1b in the paracortical areas of MLN of the infected sheep and this effect was greater in those necropsied 14 weeks after first infection, compared to those only 7 weeks post-infection. This may be an *in vivo* example of CD1b down-regulation during *M. a. paratuberculosis* infection that may not have been apparent in the previous study only 4 weeks post-infection. Such down-regulation may be an important factor in the continued survival and multiplication of *M. a. paratuberculosis* in the face of developing specific adaptive immunity.
CD45R is expressed on B cells, although B cells in ileal PP of sheep express this antigen at low density compared to peripheral B cells. A subset of T cells (naive T cells) also express CD45R. No changes in numbers of CD45R⁺ cells attributable to infection or time were found in this study using the IPX method. Using a different technique (flow cytometry) a decrease in B cell numbers in intestinal PP in early \textit{M. a. paratuberculosis} infection was demonstrated using the same monoclonal antibody. The failure to demonstrate an effect of infection on CD45R expression in this study may be due to lack of discrimination using IPX when very large numbers of labelled cells are present, precluding accurate counting. In the sub-tissues which were accurately counted and analysed in this study, the concurrent labelling of a subset of T cells may have confounded the results with respect to B cells only.

\textit{M. a. paratuberculosis} uses host physiological processes for uptake into the PP of the intestine particularly of the TI where the greatest levels of macromolecular uptake have been demonstrated. The earliest lesions of Johne's disease are usually seen in the TI and later it is the location of the most severe lesions, particularly in sheep. The ileal PP of ruminants reaches its greatest development soon after birth and involutes from about 6 months of age. Anatomically it contains abundant lymphoid follicles (mainly B cells) with little parafollicular tissue (mainly T cells) and functionally it is the major site for B-cell development and production. Previous studies have indicated that the proportion of T cells in the ileal PP taken as a whole is only 1-2% compared to 30-50% in the jejunal PP (Miyasaka et al, cited by Lugton). Although not specifically designed to examine differences between the TI and jejunal PP, the findings from this study reinforce that the terminal ileal PP in the young ruminant is very different from the jejunal PP. In this study significantly fewer CD4⁺ and CD8⁺ T cells were present in the interfollicular zones of the ileal PP on a per area basis. When one considers that the area occupied by the interfollicular zone of the ileal PP is very much less than that in the jejunal PP the difference in total numbers is very large and consistent with previous findings. This relationship was also shown to hold for \(\gamma\delta\) T cells labelled for either TCR-\(\gamma\delta\) or WC1. For WC1 this difference was observed in the overlying mucosa as well as within the lymphoid tissue. T helper (CD4⁺) cells and cytotoxic (CD8⁺) T cells are central to the adaptive immune response in mycobacterial infection, while the \(\gamma\delta\) T cells may be important in the innate immune response as well. The ileal PP of the young ruminant is relatively deficient in all of these CMI effector cells, suggesting that \textit{M. a. paratuberculosis} may
find in the ileal PP a permissive environment for the establishment and persistence of infection. In addition this study indicated that there were also fewer CD45R+ cells (mainly B cells) in the extra-follicular areas of the ileal PP, which may further compromise the development of local CMI. B cells can act as antigen-presenting cells, and have been shown to have a role in resistance to mycobacterial infection in mice, and possibly in resistance to clinical paratuberculosis in cattle. This study also indicated a difference with respect to the innervation of the area in that there were significantly more CD56+ fibres in the follicular margins of the ileal PP, but whether this may relate to mycobacterial resistance within the tissue is not clear. Taken together these findings add weight to the contention that the anatomical and immunological particularities of the ileal PP of the young ruminant may contribute to both the susceptibility of ruminants to paratuberculosis and to the development of age-related resistance to infection.
Chapter 6. A preliminary study of possible genetic influences on the susceptibility of sheep to Johne's disease

Summary
Polymorphisms at loci in a number of genes associated with immune function (NRAMP, MHC complex, IFN-γ, lysozyme, leukaemia inhibiting factor) were examined in two independent flocks of Merino sheep, each with a high prevalence of Johne's disease. Possible associations of NRAMP and MHC alleles with resistance to Johne's disease were detected.

Introduction
There has been surprisingly little specific work on the genetics of paratuberculosis resistance in ruminants. Genetic influence on the development of Johne's disease was first suggested by the observation of disease in families of cattle. However, a recent extensive study of pedigree records found only a small genetic influence on the susceptibility of cattle to M. a. paratuberculosis. There is one reference suggesting involvement of MHC alleles in the susceptibility to Johne's disease in cattle. Very little work has been published for sheep. As part of a behavioural study in sheep the CMI (lymphocyte stimulation assay) and humoral (ELISA) immune responses of two different sheep breeds after vaccination with M. a. paratuberculosis were shown to be significantly different. A preliminary study in sheep failed to find any evidence of the mutation in the NRAMP gene that leads to increased susceptibility in mice. However, studies in other species and of other mycobacterial diseases suggest that significant genetic influences on susceptibility of ruminants to paratuberculosis are likely.

Recent comprehensive breeding studies in red deer have indicated significant genetic influences on the susceptibility to tuberculosis, with high heritability. Similar studies, which might reveal genetic influences in ovine Johne's disease, are impractical because of the prolonged period of subclinical infection in infected animals and the difficulties in detecting and quantifying such infection.

For many years the susceptibility of mice to infections with a number of intracellular pathogens (Leishmania, BCG and Salmonella) has been known to be influenced by the gene coding for natural resistance associated macrophage protein (NRAMP1). A mutation making the gene non-functional is responsible for susceptibility. Mice homozygous for this mutant NRAMP allele have been shown repeatedly to be more susceptible to experimental M. a. paratuberculosis infection than wild-type mice. Similarly, major functional mutations in a wide range of
genes coding for specific cytokines or their receptors have been shown to be important in the resistance to mycobacterial diseases in both humans and laboratory animals. For example, Mendelian susceptibility to poorly virulent mycobacterial species in man has been shown to be genetically heterogeneous, a result of different types of mutations in four genes (IFNGR1, IFNGR2, IL12B, IL12RB1). The distinct disorders are immunologically related, as impaired IFN-γ-mediated immunity is the common pathogenic mechanism. Such major functional mutations in humans often lead to lethal mycobacterial infections in early childhood, and in laboratory animals provide models for pathogenesis studies. Because of the severe effects of these mutations, they are relatively easy to identify. These severe functional mutations are, however, unlikely to be of significance on a population wide level. Rather, subtle functional effects of different alleles of many different genes are likely to be involved.

In humans a number of approaches have been used to search for such genetic effects, including genome-wide screens on affected sib-pairs and the candidate gene approach. Such techniques have revealed several genetic associations with disease susceptibility that have possible relevance in the study of Johne's disease in ruminants. Polymorphisms in human NRAMP1 have been shown to be associated with both tuberculosis and leprosy. HLA (human lymphocyte antigen = MHC) class II alleles have been implicated in susceptibility to tuberculosis, leprosy and M. avium infections. Of particular interest is the association of immunopathology in leprosy with MHC class II haplotype. DQ1 is almost specific for the lepromatous form of the disease, while DR2/DR3 have been associated with the tuberculoid forms. A similar range of pathology is seen in ovine Johne's disease. There is also evidence for genetic susceptibility to the inflammatory bowel condition Crohn's disease in which M. a. paratuberculosis has a disputed role, and recently a specific association of variants of the NOD2 gene which influence monocyte function have been demonstrated to confer susceptibility to this condition. Other reports include an IL-1 gene cluster association with tuberculosis, association of functional mutant homozygotes for mannose binding protein with pulmonary tuberculosis, and an association of the vitamin D receptor gene with both susceptibility to tuberculosis and type of pathology in leprosy. Complementary studies have shown effects of genotype for MHC, vitamin D receptor and mannose binding protein on CMI responses in tuberculous patients.

The candidate gene approach is applicable to the investigation of genetic influences on the susceptibility of sheep to Johne's disease. When applied to human populations differences in the frequencies of alleles of known genes between affected and racially and ethnically matched control individuals denote functional significance of those genes or linkage to some other functionally significant gene nearby. In sheep comparisons between affected and unaffected sheep within flocks serve the same function. Sheep of similar age within a traditionally managed flock share a similar genetic background and experience similar husbandry and environmental
conditions. In particular they have had the same degree of exposure to the same strains of *M. a. paratuberculosis*. A number of polymorphic loci for genes coding for various aspects of immune function have been reported in sheep and to date none have been systematically examined for possible influence on the development of OJD (ovine Johne's disease). Loci shown to be involved in resistance to mycobacterial infections in man (above), and others known to be critical in the pathogenesis of mycobacterial infections are obvious candidates for investigation.

In this study we examined two flocks of sheep from 2 farms which had been experiencing significant losses due to Johne's disease over several years, so that environmental contamination with *M. a. paratuberculosis* was high. Sheep were classified on the basis of clinical, pathological, cultural and immunological tests as susceptible or resistant to Johne's disease, and correlations were sought with genotype for a range of immune function genes.

**Methods**

**Experimental flocks:**

Two flocks of fine-wool merino sheep from the southern tablelands of New South Wales with a history of significant on-going losses from Johne's disease were examined.

Flock A originated from a farm that had experienced annual mortalities of about 10% due to OJD, and had been purchased specifically for other OJD research. The disease was suspected to have been present on the original farm for at least 8 years. 12.7% of 345 ewes were seropositive in the AGID, and faecal culture results were available for most sheep. 119 adult ewes were selected on the basis of faecal culture and serology (65 animals positive on one or both tests and 54 negative on both) for further examination. Selected sheep were then examined at a single point in time, but due to ongoing losses only 106 were available for detailed examination. Sheep were skin tested then sent to slaughter one week later, except for six considered too ill for commercial slaughter which were necropsied in the field.

Flock B was from a farm that had experienced annual mortalities of up to 20% due to OJD and that had been the subject of on-going Johne's disease research. The disease was suspected to have been present on the farm for at least 8 years. Seropositivity amongst random groups of 50 to 133 clinically normal sheep 2-3 years of age sampled on four occasions between 1998 and 2000 ranged from 8 to 16%. Most of the sheep classified as susceptible in the current study were examined at different times over a period of several years when euthanased during other studies. Skin testing was not done on these. Most of the samples for possibly resistant animals were obtained when a cohort of 50 remaining sheep (by then all at least 4 years old) from the
flock were sampled together (AGID, faecal culture and skin testing). It was not possible to obtain samples for pathology from these latter sheep.

**ASSESSMENT OF CLINICAL SIGNS:**

Sheep were classified as having clinical signs of Johne's disease on the basis of severe emaciation, with or without diarrhoea, and no other concurrent disease or nutritional cause.

**SEROLOGY FOR ANTIBODIES TO M. A. PARATUBERCULOSIS:**

Plain blood samples were allowed to clot and retract, and serum removed and stored at -20 °C for up to 4 months until assayed in the AGID. A reaction of one, two or three plus was considered positive, and a negative or inconclusive reaction classed as negative.

**INTRADERMAL TESTING FOR DELAYED HYPERSENSITIVITY:**

Sheep were injected intradermally on the wool-free inner thigh with 0.1 mL of Avian tuberculin PPD (25000 units per mL CSL, Parkville, Victoria). Skin thickness was measured with vernier callipers before injection and 72 hours later. A skin thickness increase of > 4mm was regarded as positive, ≤ 4mm as negative.

**NECROPSY SAMPLING:**

Severely clinically affected sheep were euthanased with IV barbiturate. Other sheep were sampled during routine abattoir slaughter. The ileocaecal valve (ICV) and terminal metre of ileum, with attached mesentery and nodes were examined in detail. Enlarged mesenteric nodes, lymphangitis or thickened ileal mucosa were regarded as positive signs for ovine Johne's disease in the gross necropsy. Samples of terminal ileum, ICV and mesenteric nodes were collected into buffered neutral formalin for histopathology. Terminal ileum and MLN were collected for culture.

**M. A. PARATUBERCULOSIS ISOLATION:**

Faeces or tissues were stored frozen at -80 °C for up to 12 months until processed using routine methods as previously described (Chapter 2). Positive culture results were listed as ++ (growth index exceeded 999 in ≤ 4 weeks) or + (growth index exceeded 999 in > 4 weeks).

**HISTOPATHOLOGY:**

Tissues were processed routinely for histopathology, sectioned at 5 μ, stained with haematoxylin & eosin (H&E) and a Ziehl-Neelsen technique (ZN), and examined by light microscopy. Lesions were scored as sm (severe/diffuse multibacillary), sp (severe/diffuse paucibacillary), f (mild, focal or multifocal lesions) or 0 (no lesions consistent with Johne's
disease). Presence of acid-fast bacilli (AFB) was scored as ++ (many present in most fields), + (few present) or 0 (none observed in any field).

**Phenotypic classification of sheep:**

**Observed disease status of individual sheep:** Sheep were classified as severely affected, mildly affected or as unaffected on the basis of faecal culture results, clinical and necropsy findings.

* **Severely affected sheep** had at least one of:
  * severe clinical Johne's disease (died or euthanased due to Johne's disease)
  * diffuse or multifocal pathology with abundant AFB
  * faecal culture positive and excreting very large numbers of organisms (gi 999 ≤ 4 wks)

* **Mildly affected sheep** had none of the above and at least one of:
  * diffuse, focal or multifocal pathology with few AFB
  * faecal culture positive, with low or moderate numbers of organisms (gi 999 > 4 weeks)
  * tissue culture positive

* **Unaffected sheep** had none of the above signs, ie were not clinically affected, had no significant pathology, and were negative on faecal and tissue culture.

**Overall assessment of susceptibility/resistance (Flock A):** This assessment was designed to make use of all available data for an individual sheep including specific CMI and humoral immune status as well as the observed disease status.

* **s: susceptible sheep** - severe observed disease and/or serologically positive
* **ds: doubtful but possibly susceptible sheep** - mild observed disease, CMI negative and seronegative
* **dr: doubtful but possibly resistant sheep** - mild observed disease, CMI positive and seronegative
* **rr: resistant sheep** - no evidence for disease, CMI positive and seronegative
* **ri: innately resistant sheep** - no evidence for disease or infection, CMI negative and seronegative

**Overall assessment of susceptibility/resistance (Flock B):** As it was not possible to perform all tests on most sheep in this flock, only two categories of resistance were possible.

* **s: susceptible sheep** - those with severe observed disease and/or serologically positive
* **r: possibly resistant** - all other sheep.
CANDIDATE GENES:

Loci with known polymorphisms in the following genes were investigated using PCR to amplify DNA fragments.

NRAMP: OVIRNA1, a microsatellite, dinucleotide repeat on chromosome 2, amplifying a 160 bp fragment, with 8 alleles reported. Primer sequences used were GCC ACG GGT GGG ATG AGT (forward) and TGA GCT AGG AGA TAG CAG G (reverse).

MHC complex: CSRD226, a microsatellite, dinucleotide repeat on chromosome 20, amplifying a 149 to 195 bp fragment, with 18 alleles reported. Primer sequences used were TGG AGA ATT CCA TGG TTA GAG GAG (forward) and GAT GGC TGG AAG CAG ATA CTC TAA (reverse).

Lysozyme: LYZ, a microsatellite, dinucleotide repeat on chromosome 3, amplifying a 192 to 209 bp fragment, with 5 alleles reported. Primer sequences used were GCA CCA GCA GAG AGG ACA TT (forward) and ACC GGC TAT TGT CCA TCT TG (reverse).

Interferon gamma: IFNG, a microsatellite, tetranucleotide repeat on chromosome 3, amplifying a 124 to 128 bp fragment, with 2 alleles reported. Primer sequences used were TTG TGA CTG TTA GCT AGA TGT GTT (forward) and ATA CAC ATA TTA TGC CCA TCT TTT (reverse).

Leukaemia inhibiting factor: LIF, a microsatellite, dinucleotide repeat on chromosome 17, amplifying a fragment of about 104 bp, with 9 alleles. (Jill Madison, personal communication) Primer sequences used were CTG CAG GGC AAG TGA TT G GAT T (forward) and TCA GCC CTT GGG CGT CAG T (reverse).

DNA EXTRACTION:

Liver or lymph nodes were collected at necropsy and held frozen at -20 ºC until processed. EDTA blood samples were centrifuged and the buffy coat collected. Remaining red cells were removed by osmotic lysis in sterile water and the resulting white cell pellet held frozen at -20 ºC until processed. Sheep DNA was extracted from approximately 100mg of tissue or cell pellets using DNAzol reagent (Life Technologies) according to manufacturer’s instructions. Extracted DNA was dissolved in 8mM NaOH, quantified by absorbance at 260 nm and diluted in water to 5 μg/mL for use in PCR.

PCR:

For PCR, 25 ng target DNA was amplified in a 10μL reaction, comprising 100μM of each dNTP, 1 μM of each primer, 1.5 mM MgCl₂, 67mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/mL gelatin, and 0.1 units of Taq. α³²P d-CTP (0.1 μL of a
solution containing 10μCi/μL was included in each reaction. Reactions were performed in 96 well microtitre trays and run on a DNA thermal cycler using the following protocol: 3 cycles of denaturation at 94°C for 3 min, annealing at 55°C for 20s and extension at 72°C for 10s, followed by 31 cycles of 94°C for 1min, 50°C for 40s and 72°C for 20s. PCR product was visualised on 6% polyacrylamide gel containing 40% urea by autoradiography.

Scoring the genotype from the polyacrylamide gels required some experience. Genotype for NRAMP was scored independently by 2 observers and a “consensus” agreed when the results differed initially. The following observations were considered important in scoring genotype correctly. One or more “stutter bands” are usual, are more prominent for smaller fragments and appear to be located at multiples of 2 bp below the main band. They are rarely seen above the main band (or if present are very much less intense than the bands below) although a halo without a distinct band is often present. Thus for a single isolated allele (or a homozygote) a distinct band at the nominal size, with stutter bands of decreasing intensity extending below is seen (see for example Fig 6.2b sheep T5, alleles i and r, or sheep T9, homozygous for allele m). In heterozygotes the stutter bands from one allele will often overlap the nominal band of the other allele causing increased band intensity. This is especially prominent when the alleles differ in size by only 2bp. In this case a distinct band is seen for the larger allele and a more intense band immediately below representing the combined effect of the main band for the shorter allele and the first stutter band of the larger (see for example Fig 6.5b sheep T3, genotype bc).

**Statistical Analysis:**

For each candidate gene in each flock genotypes were compared initially between different phenotype groups using the chi-square test on ‘a X b’ contingency tables using Minitab statistical software (a = number of genotypes, b = number of phenotype groups). The phenotypes considered were overall susceptibility/resistance (5 groups for Flock A, 2 for Flock B), observed disease (3 groups), histopathology (4 groups), CMI (2 groups) and humoral response (2 groups). Where chi-square results were likely to be invalid (cells with expected values less than 1) analysis was performed with respect to up to three of the most common alleles, “k”, comparing genotypes “kk”, “k/other” and “other/other” for each phenotypic classification in ‘3 X b’ tables. If too few “kk” individuals were present in the flock “kk” and “k/other” genotypes were pooled for analysis in ‘2 X b’ tables. Phenotypic groups were also pooled into dichotomous classifications for analysis in ‘3 X 2’ or ‘2 X 2’ tables as follows. With regard to overall susceptibility/resistance, comparison was made between definitely susceptible sheep (group s) and all other sheep (groups ds, dr, rr and ri), between susceptible and possibly
susceptible sheep (groups s and ds) and other sheep (groups dr, rr and ri), between sheep with any evidence for disease (groups s, ds and dr) and those with no evidence for disease (groups rr and ri), and between those with any evidence of infection (groups s, ds, dr and rr) and sheep with no evidence for infection (group ri). With regard to observed disease status, comparisons were made between sheep with no observed disease and those with any observed disease, and between sheep with no or mild observed disease and those with severe disease. With regard to pathology, additional comparisons were made between sheep with severe multibacillary pathology and sheep with mild or paucibacillary lesions, and between sheep with any histological lesions and those having no lesions.

Because of the large number of individual tests of significance performed, and also the correlations between different phenotypic categories, the probabilities obtained from particular chi-square tests could not be used as actual indicators of significance. Rather, they were used only as an empirical guide to possible associations, and such associations with P values of $\leq 0.1$ are detailed in the results section. However, when similar associations between particular genotypes and phenotype occurred independently in both flocks examined this was considered to be evidence for a likely real association. In each flock, alleles were also ranked for possible association with resistance based on the percentage of each allele associated with resistant phenotypes (phenotypes (rr + ri) for Flock A and phenotype r for Flock B). The occurrence of similar ranking in the two independent flocks was considered another indicator of possible associations.

Results
Summary results for phenotypic assessment and genotype of individual sheep in each flock are given in Appendices 6a and 6b.

In Flock A, phenotypic results were available for most tests from most sheep. Faecal and tissue cultures were often omitted from sheep with typical histopathological lesions because of financial constraints. It was possible to provide an assessment of disease status for each of the 106 sheep. 29 had no observed OJD, 54 were assessed as having mild disease and 23 as severely diseased. Skin test results were available for 105 sheep (60 negative and 45 positive) and AGID results for 105 (74 negative and 31 positive). Overall 40 sheep were classified as susceptible, 21 as possibly susceptible, 14 as possibly resistant, 11 as resistant and 20 as innately resistant. In Flock B, a detailed assessment of disease status was only possible for 45 animals (3 with no observed disease, 4 with mild disease and 38 with severe disease). Of the remaining 47
animals (all negative on faecal culture) 40 were clinically healthy at a subsequent muster 6 months after skin testing, and were classified as probably resistant. Seven were presumed to have died in the paddock, possibly of OJD. Three of these were serologically positive and so classified as susceptible. The other four were classified as “?” with regard to clinical signs and were omitted from analysis of susceptibility/resistance. Skin test results were available for 49 sheep (26 negative and 23 positive) and AGID results for 83 (53 negative and 30 positive). Overall 41 sheep were classified as susceptible and 47 as probably resistant.

Detailed results and analysis for each candidate gene are given below. Figures 6.1 – 6.5 depict typical examples of the alleles observed for each candidate gene. For most genes, valid comparisons between sheep with different types of pathology or observed disease were not possible in Flock B because few sheep with mild lesions had been necropsied.

**NRAMP alleles:**

Seven alleles designated –3, –2, 0, 1, 2, 3, 4 were identified. See Table 6.1, Figures 6.1a and 6.1b. Alleles –3, –2 and 4 were absent from Flock A, and alleles –3 and 4 were present in only one sheep each in Flock B. Allele 1 was the most frequent allele in both flocks. In Flock A there was an interaction of genotype (0/other, other/other) with observed disease status (Chi-square = 10.594, DF = 2, P = 0.005, n = 105) and with histopathology (Chi-square = 9.266, DF = 3, P = 0.026, n = 104). Genotype 0/other was associated with sheep with severe observed disease ((0+mild), severe), (Chi-square = 10.132, DF = 1, P = 0.001), and with severe multibacillary pathology (sm, f/sp), (Chi-square = 7.025, DF = 1, P = 0.008, n = 50). No associations of genotype (with respect to alleles 1 or 3) with phenotype were detected. In Flock B there was an interaction of genotype (11, 1/other, other/other) with susceptibility, (Chi-square = 4.953, DF = 2, P = 0.084, n = 80). Genotype 11 was associated with resistant phenotype (Chi-square = 3.89, DF = 1, P = 0.048). No associations of genotype (with respect to alleles 0 or 2) with phenotype were detected. Alleles were ranked for apparent association with resistance 1,2,3,0 (Flock A) and 1,-2/3,2,0,-3/4 (Flock B).

<table>
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<tr>
<th>Allele</th>
<th>Size</th>
<th>Frequency</th>
<th>Flock A</th>
<th>Flock B</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3</td>
<td>N+8</td>
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<td>&lt;0.01</td>
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</tr>
<tr>
<td>-2</td>
<td>N+6</td>
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<tr>
<td>0</td>
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<td>0.14</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>N(~160bp)</td>
<td>0.44</td>
<td>0.35</td>
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</table>
Figure 6.1a. NRAMP alleles, Flock A

Sheep ID: 13 14 15 16 17 18 19 20
Genotype: 11 23 03 01 13 12 13 11

Figure 6.1b. NRAMP alleles, Flock B

Sheep ID: t2 t3 t4 t5 t6 t7
Genotype: 12 -22 01 none -23 22
MHC complex:

Thirteen alleles arbitrarily designated i,j,l,m,n,o,p,q,r,t,u,v,w were identified in the sheep examined. See Table 6.2, Figures 6.2a and 6.2b. Alleles j, u and w were absent from Flock A, and alleles p, t and v were absent from Flock B. Allele r corresponds to the reported most frequent allele of 163 bp. Allele m was the most frequent allele in both flocks examined in this study. In Flock A there was an interaction of genotype (mm, m/other, other/other) with observed disease (none, mild/severe), (Chi-square = 5.989, P = 0.05, n = 98). Genotype mm was associated with absence of observed disease (Chi-square = 5.9, DF = 1, P = 0.015), and with absence of histological lesions (Chi-square = 4.04, DF = 1, P = 0.04, n = 97). There was also an interaction of genotype (rr, r/other, other/other) with susceptibility (s, ds/dr/rr/ri), (Chi-square = 6.612, DF = 2, P = 0.037), with observed disease (Chi-square = 8.601, DF = 4, P = 0.072), and with serological status (Chi-square = 8.681, DF = 2, P = 0.013, n = 97). Genotype rr was associated with susceptibility (Chi-square = 3.772, DF = 1, P = 0.052), with the presence of histological lesions (Chi-square = 5.16, DF = 1, P = 0.023) and with seropositivity (Chi-square = 6.31, DF = 1, P = 0.012). Genotypes tt and t/other (pooled) were associated with susceptibility (s, ds/dr/rr/ri), (Chi-square = 3.172, DF = 1, P = 0.075), with severe disease (Chi-square = 2.909, DF = 1, P = 0.088) and with multibacillary pathology (Chi-square = 6.402, DF = 1, P = 0.011, n = 49). In Flock B there were no sheep with the rr genotype, but genotype r/other was associated with susceptibility (Chi-square = 4.528, DF = 1, P=0.033, n = 63) and seropositivity (Chi-square = 4.017, DF = 1, P=0.045, n = 55). There were no associations of genotype (with respect to alleles m or o) with phenotype. Alleles were ranked for apparent association with resistance o,t,m,n,r,q (Flock A) and q,l,o,w,m,j,n,u,r (Flock B).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Size</th>
<th>Literature Frequency</th>
<th>Flock A Frequency</th>
<th>Flock B Frequency</th>
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<tr>
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<td>u</td>
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<td>v</td>
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**Figure 6.2a. MHC complex alleles, Flock A**

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<td>mm</td>
<td>mm</td>
<td>mr</td>
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**Figure 6.2b. MHC complex alleles, Flock B**

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<td>ir</td>
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169
LYSOZYME ALLELES:

Five alleles, designated a,b,c,d and e were identified and were present in each flock. See Table 6.3, Figure 6.3. Allele e, the reported most frequent allele was also the most frequent in each flock. In Flock A there was an interaction of genotype (cc, c/other, other/other) with susceptibility (s, ds/dr/rr/ri) (Chi-square = 7.589, DF = 2, p = 0.022, n = 102), and with observed disease (Chi-square = 11.071, DF = 4, p = 0.026, n = 102). Genotype cc was associated with susceptibility (Chi-square = 7.06, DF = 1, P=0.008), with severe observed disease (Chi-square = 6.30, DF = 1, P=0.012), with the presence of histological lesions (Chi-square = 2.849, DF = 1, P = 0.091, n = 101) and with seropositivity (Chi-square = 3.76, DF = 1, P = 0.052, n = 101). Genotypes ee and e/other (pooled) were associated with the absence of histological lesions (Chi-square = 3.39, DF = 1, P = 0.066). No association of genotype (with respect to allele d) with phenotype was detected. In Flock B no associations of genotypes (with respect to alleles c, d or e) with phenotype were detected, and there were no sheep with genotype cc. Alleles were ranked for apparent association with resistance b,d,e,c,a (Flock A) and c,d,b,c,a (Flock B).

Table 6.3. Lysozyme. Alleles at the LYZ locus

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</tbody>
</table>
IFN-γ ALLELES:

The two reported alleles, designated 1 and 2, were identified and were present in both flocks. See Table 6.4, Figure 6.4. In Flock A there was an interaction of genotype with observed disease (none/mild, severe), (Chi-square = 6.149, DF = 2, P = 0.046, n = 105). Genotype 22 was negatively associated with severe disease (Chi-square = 4.47, DF = 1, P = 0.035, n = 105). No associations of genotype with any phenotypic character were detected in Flock B. Alleles were ranked for apparent association with resistance 1,2 (Flock A) and 2,1 (Flock B).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Size</th>
<th>Frequency Literature</th>
<th>Flock A</th>
<th>Flock B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128</td>
<td>0.41</td>
<td>0.57</td>
<td>0.33</td>
</tr>
<tr>
<td>2</td>
<td>124</td>
<td>0.59</td>
<td>0.43</td>
<td>0.67</td>
</tr>
</tbody>
</table>
LEUKAEMIA INHIBITING FACTOR ALLELES:

Eight alleles were identified. These were designated a, b, c, d, e, f, g and i. See Table 6.5, Figures 6.5a and 6.5b. The g and i alleles were not present in Flock A, and allele a was absent from Flock B. Allele e was the most frequent allele in both flocks. In Flock A there was an interaction of genotype (ee, e/other, other/other) with possible susceptibility (s/ds, dr/rr/ri), (Chi-square = 11.941, DF = 2, P = 0.003, n = 102), with evidence for disease (s/ds/dr, rr/ri), (Chi-square = 8.868, DF = 2 , P = 0.012), with evidence for exposure (s/ds/dr/rr, ri), (Chi-square = 5.192, DF = 2 , P = 0.052), and with observed disease (none, mild/severe), (Chi-square = 6.148, DF = 2, P = 0.046, n = 102). Genotypes ee and e/other (pooled) were associated with possible susceptibility (Chi-square = 11.37, DF = 1, P < 0.001), with evidence for disease (Chi-square = 8.82, DF = 1 , P = 0.003), with evidence for exposure ( Chi-square = 4.05, DF = 1, P = 0.044) and with observed disease (Chi-square = 6.14, DF = 1, P = 0.013). No associations of genotype (with respect to alleles b or c) with phenotype were detected. In Flock B genotypes cc and c/other (pooled) were associated with susceptibility (Chi-square = 2.71, DF = 1, P = 0.1, n = 67), with severe disease (none/mild, severe), (Chi-square = 4.81, DF = 1, P = 0.028, n = 44) and with positive DTH (Chi-square = 3.475, DF = 1, P = 0.062, n = 28). There was also an interaction of genotype (ee, e/other, other/other) with susceptibility (Chi-square = 6.637, DF = 2, P = 0.036). Genotypes ee and e/other (pooled) were associated with resistance (Chi-square = 5.34, DF = 1, P = 0.02). No association of genotype (with
respect to allele b) with phenotype was detected. Alleles were ranked for apparent association with resistance d,f,c,b,a,e (Flock A) and b,e,d,c,f,g (Flock B).

Table 5. Leukaemia inhibiting factor. Alleles at the LIF locus

<table>
<thead>
<tr>
<th>Allele</th>
<th>Size</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flock A</td>
<td>Flock B</td>
</tr>
<tr>
<td>a</td>
<td>N+8</td>
<td>0.07</td>
</tr>
<tr>
<td>b</td>
<td>N+6</td>
<td>0.25</td>
</tr>
<tr>
<td>c</td>
<td>N+4</td>
<td>0.09</td>
</tr>
<tr>
<td>d</td>
<td>N+2</td>
<td>0.05</td>
</tr>
<tr>
<td>e</td>
<td>N (~104bp)</td>
<td>0.45</td>
</tr>
<tr>
<td>f</td>
<td>N-2</td>
<td>0.09</td>
</tr>
<tr>
<td>g</td>
<td>N-4</td>
<td>0</td>
</tr>
<tr>
<td>i</td>
<td>N-8</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 6.5a. Leukaemia inhibiting factor alleles, Flocks A and B
Discussion

Traditional selection of sheep for resistance to Johne's disease is unlikely ever to be practical. Resistant animals can best be identified near the end of their reproductive life, and then only if kept in a highly contaminated environment since birth. Such management is unlikely to be possible in a commercial sheep stud. However, if specific alleles of known genes which confer some resistance to the development of Johne's disease could be identified, then rams identified as possessing those alleles could be used in severely affected flocks in a cost-effective manner.

A major problem for investigations into the genetics of paratuberculosis resistance in ruminants is the difficulty in accurately identifying susceptible or resistant animals. Due to the focal or segmental nature of Johne's disease pathology, the lack of consistent gross lesions (especially in sheep) and the limited amount of tissue that can practically be examined by histopathology, some lesions will be missed even during a thorough necropsy. The problems are compounded when one seeks to determine disease status or susceptibility in the live animal. Sampling effects, intermittent shedding of the organism and the effects of necessary decontamination procedures for culture mean that some false negative faecal and tissue cultures are to be expected. Conversely, occasional uninfected animals will have positive faecal cultures due to passive excretion of ingested organisms. Moreover simple infection with *M. a. paratuberculosis* needs to be differentiated from actual disease. For example, tests for CMI such as the IFN-γ test have shown some promise in identifying infected sheep, especially
in the early stages. However, some infected animals with the largest IFN-γ responses later had no evidence of clinical disease.\textsuperscript{131} If selection based on IFN-γ response were used to remove infected animals from a flock, many possibly very resistant sheep would be removed also. While serological tests for Johne's disease have low sensitivity, their specificity is high and they may be useful for identifying at least some susceptible animals.\textsuperscript{256,324}

Large numbers of animals are needed to detect small differences in susceptibility due to specific gene loci. For example, using the candidate gene approach, to have a 80\% power to detect (P<0.05) an effect of a susceptibility allele with a population frequency of 0.5 and exerting a 2 fold increase in susceptibility would require 340 cases.\textsuperscript{29} Limitations in the number of sheep available for the study of resistance to Johne's disease are due to economics and the nature of the disease. No one test indicates disease state in a sheep and culture for \textit{M. a. paratuberculosis} is expensive to apply to large numbers of individual animals. Similarly, detailed necropsy with histopathology is expensive, and the very extended period between infection and emergence of disease means experimental exposure is usually too expensive and impractical to be used for genetics studies. In this study, even by working with on-going research projects only about 100 animals in each of two flocks could be investigated in any detail. Thus small effects due to specific genes would be unlikely to be revealed by this investigation.

Because no one test or even combination of tests is 100\% effective at identifying infected or diseased sheep, it was important to examine the phenotypic data from many viewpoints and a variety of phenotypic categories were used. The results of a range of tests – clinical and pathological findings and isolation of \textit{M. a. paratuberculosis} from both faeces and tissues - were used to obtain the best objective assessment of the “observed disease status”. This three point classification (none, mild and severe) was considered more reliable than applying data from specific tests in isolation. For example, sheep excreting very large numbers of organisms are likely to have multibacillary lesions, and the use of quantitative estimates for faecal culture allowed better classification of some sheep for which necropsy data were not available, or where focal or segmental lesions may have been missed during abattoir slaughter. Note that sheep with mild disease are not necessarily susceptible sheep – they may have arrested lesions and effective immunity. Histopathological results were, however, also used in isolation both to examine for genetic influences on the type of pathology (paucibacillary or multibacillary), and because this test has traditionally been used as the “gold standard” in assessing other tests. In taking observed disease status further to attempt to assign susceptibility or resistance to
individual sheep the immunological responses of the sheep were considered. Severely diseased sheep (regardless of observed immunity) were considered unlikely to recover and were classified as susceptible (group s). Humoral antibodies are not protective in mycobacterial infections, rather an indicator of the failure of containment of infection. The AGID has very high specificity for Johne's disease, and moreover positive AGID results are highly correlated with diffuse pathologic changes and large numbers of AFB in the tissues. So sheep with positive AGID results were assessed as susceptible animals. This allowed classification of several sheep in Flock A with mild observed disease as susceptible animals, and also a single sheep with no observed disease. This was considered reasonable because lesions above the terminal ileum would have been overlooked during abattoir slaughter. In Flock B several sheep for which no pathology was available could also be classified as susceptible. Cell mediated immunity is the more important protective immune response against \textit{M. a. paratuberculosis}. Sheep with focal and/or paucibacillary lesions tend to have positive DTH reactions, whereas sheep with severe disease and large numbers of AFB in tissues tend to be negative. Thus for sheep with other evidence for infection positive DTH is indicative of resistance while a negative test points towards susceptibility. Mildly affected sheep with positive DTH were classified as doubtful but probably resistant (group ‘dr’) and those with negative DTH as doubtful but possibly susceptible (group ”ds”). Sheep with no observed disease (and therefore likely to be resistant sheep) were also subdivided on the basis of their DTH reactions. Sheep with negative DTH were considered innately immune (group ri). Innate immunity which includes macrophage function, possibly aspects of gut physiology such as M cell function, and even behavioural traits, may play an important role in the resistance of sheep to Johne's disease. In sheep with no observed disease and positive DTH (group rr) \textit{M. a. paratuberculosis} infection presumably reaches sufficient level to trigger adaptive responses. A high specificity (100\%) has been claimed for DTH tests in sheep using histopathology as a reference, but this was not the case in this study. In Flock A, of 52 sheep histologically negative for OJD, only 36 were negative in the skin test, yielding an apparent specificity of only 67\%.

The use of sheep from highly infected flocks (>10\% annual losses) was critical for this study. Clinically affected sheep excrete very large numbers of \textit{M. a. paratuberculosis} organisms (> 10^8 per g faeces), and it is reasonable to assume that sheep grazing in continuous contact with such animals have been exposed to significant numbers of \textit{M. a. paratuberculosis} many times. Thus aged healthy sheep from these flocks are very likely to be resistant animals.
NRAMP:

The role of NRAMP in resistance to intracellular pathogens appears to be mediated by its involvement in transporting iron out of infected phagosomes of macrophages. In mice differences in susceptibility due to NRAMP are only seen when macrophages are activated by either non-specific or immunologically specific stimuli. A preliminary study failed to find any evidence of the mutation in the NRAMP gene that leads to increased susceptibility in mice. This is not unexpected because the particular mutation has been shown to be the same single base substitution in all strains of Bcg mice and considered to be identical by descent. In this study a possible association of allele 0 with severe disease and multibacillary pathology was detected in Flock A, and in Flock B the 0 allele was ranked of the common alleles as the most associated with susceptibility. It is also interesting that no homozygotes for this allele were seen in Flock A (P = 0.03) and only a single homozygote in Flock B, despite allele frequencies of 14 and 18%. All sheep in this study were adults. If homozygous 00 sheep did have poor NRAMP function they may have succumbed at a young age to OJD or other infectious disease. There was also some suggestion that allele 1 may be associated with resistance. In Flock B genotype 11 was associated with resistance and in both flocks allele 1 was ranked as the most associated with resistance.

MHC:

The MHC genes are closely linked and usually inherited as a haplotype. MHC class I molecules are present on the membrane of all nucleated cells and present antigens to cytotoxic T cells. MHC class II molecules are mainly located on the cells of the immune system and present antigens to helper T cells. Depending on the antigen or epitope presented these responses could lead to protective immunity to DTH or to immune suppression. Located between the class I and II genes (in humans at least) are genes for complement factors and TNF all of which are potentially important in immune function. As expected polymorphisms in the MHC genes have a role in susceptibility to many disease conditions including the mycobacterial diseases, and a possible association of particular MHC haplotypes with Johne's disease in cattle has been reported. In this study there was some evidence that allele r may be associated with susceptibility to OJD. An association of genotype rr with susceptibility was observed in Flock A, and genotype r/other with susceptibility in Flock B. And in both flocks allele r was not the most frequent allele, although reported to be so in the literature. This might represent natural selection within these heavily diseased flocks against susceptible animals, or may simply be a reflection of the allele frequencies in fine-wool merinos. Also of interest also is the fact that the frequency of allele r in Flock B was half that of Flock A. Flock B has used home bred rams for
many years, whereas Flock A buys rams each year from one apparently OJD-free stud. Thus any natural selection for resistance would have been stronger in Flock B. Moreover, of the common alleles, allele r was ranked as the most associated with susceptibility in both flocks. In fact the ranking of the most common alleles (o,m,n,r) for association with resistance was identical in both flocks. Possible associations of genotype mm with absence of disease or histological lesions and allele t with susceptibility, severe disease and multibacillary pathology were seen in Flock A only.

LYSOZYME:

Lysozyme is synthesised by paneth cells at the base of the intestinal crypts, and is an important antibacterial component within phagolysosomes of macrophages. Its antibacterial action involves the degradation of the peptidoglycan within bacterial cell walls. There was little evidence from this study for an effect of polymorphism at this locus on susceptibility to Johne's disease. A possible association of genotype cc with disease, susceptibility and seropositivity was seen in Flock A, but the frequency of allele c in Flock B was too low for any effect to be seen.

IFN-γ:

This is the major cytokine involved in macrophage activation and resistance to *M. a. paratuberculosis*, and allelic variations in the gene coding for this protein might have significant functional effects. In man functional mutations in several genes influencing IFN-γ function are associated with increased susceptibility to mycobacterial infections. In the present study in sheep there was little evidence that polymorphisms at the IFNG locus affected susceptibility to Johne's disease. Genotype 22 appeared to be protective in Flock A, but no such association was seen in Flock B, and allele 2 was ranked as more “susceptible”.

LEUKAEMIA INHIBITING FACTOR:

In some strains of mice expression of primarily Th1 or Th2 cytokines after *M. avium* infection has been shown to be genetically controlled, independent of the MHC complex and NRAMP status. There is some evidence that LIF is one gene that may be involved in this balance between Th1 and Th2 immune function. It has been shown to stimulate the production of inflammatory cytokines, and to be important in the maintenance of pregnancy by its association with the local Th2 milieu of the placenta. No investigations for its role in disease resistance in ruminants appear to have been published to date, although a role in pregnancy maintenance in these species is likely. In the present study there was little evidence that polymorphism at the LIF locus influenced resistance to Johne's disease. An association of allele
e with possible susceptibility, evidence for disease or infection and with observed disease was seen in Flock A. However the opposite effect was seen in Flock B with allele e apparently associated with resistance. A possible association of allele c with susceptibility, severe disease and (paradoxically) positive DTH was seen in Flock B only. The ranking of alleles for susceptibility also showed no similarity between the two flocks.

In conclusion this preliminary study has revealed possible associations of polymorphisms at the NRAMP and MHC loci with susceptibility to Johne's disease in sheep, but no consistent effects at the other loci were found. It is of interest to note that the findings using serological data only compared to more exhaustive analysis using culture and histopathology were similar for MHC and lysozyme. This suggests that screening large numbers of sheep from infected flocks using serology and automated genotyping might be a practical alternative for further preliminary investigations. Further studies into the genetics of resistance to ovine Johne's disease are warranted.
Chapter 7. General discussion and conclusions

The chronic and insidious nature of the disease process in *Mycobacterium avium* subsp. *paratuberculosis* (M. a. paratuberculosis) infection means that experimental investigation in ruminants is often difficult and expensive. Although some laboratory animal models for Johne's disease are available, the much greater susceptibility of ruminant species to *M. a. paratuberculosis* infection means that studies in laboratory animals need to be interpreted with caution. The study of ovine Johne's disease (OJD) in particular has been further hampered by the historical difficulties in culturing the ovine strains of *M. a. paratuberculosis*. The studies described in this thesis were undertaken against the background of an industry need to understand OJD in the Australian context, and in particular a desire to better understand and diagnose early infection in field situations. Thus funding was available to examine the early pathogenesis of OJD using experimental infection in the ruminant host with low doses of ovine strains of *M. a. paratuberculosis*, studies which were likely to be more representative of natural infection than most previous studies. And follow-up field studies, also looking specifically at early infection, were possible. The current studies were unique in this regard.

The reliable culture of ovine strains of *M. a. paratuberculosis* has only been possible recently, and this study was able to take advantage of this in the investigation of the pathogenesis of OJD. Most previous experimental infections of sheep have used isolates with characteristics of bovine strains. There may be significant strain differences in response to experimental infection. For example, early work in the UK demonstrated that there was a considerable increase in the numbers of organisms (bovine strains) in the intestinal tissues over the numbers originally administered in the first few months after infection. Such was not the case in the pen trials in the present study using ovine strains. Another difference between this study and studies with bovine strains was the lack of early specific histological changes in confirmed-infected lambs, both in pen trials and in the field, whereas lesions consisting of clumps of epithelioid macrophages within the intestinal PP have frequently been described in lambs examined in the first few months after experimental infection with bovine strains. This difference may also be associated with the higher doses used in most earlier studies (see below), although some of the lambs with reported lesions had received low doses. Australian Merinos might also respond differently to the mainly British breeds used in other studies.

Dose rates may have a profound effect on the outcome of *M. a. paratuberculosis* infection. In a series of studies with bovine strains in the 1960's, Brotherston *et al* demonstrated that very high
doses (>10⁹ viable units) led to early progressive infection which was not typical of the natural situation, whereas lower doses (10⁶ or 10⁷) did not. Despite these early reports, most subsequent experimental infections of sheep with *M. a. paratuberculosis* have used very high doses. The use of very high doses in many experimental infections is perhaps understandable. Johne’s disease research is a very long term and a very expensive undertaking. Using too low a dose could mean no infected animals several years into a project – disastrous when continued funding depends on some positive results! However, the high-dose, bovine-strain infection approach may not be representative of the usual infection of sheep in natural situations, so that data generated from such trials, eg. the performance of diagnostic tests, should be viewed with caution. Studies with more natural dose levels, as in the current research, may assist in the interpretation of findings from other high-dose studies.

Because the dose of *M. a. paratuberculosis* can have an effect on the outcome of infection, techniques for the enumeration of *M. a. paratuberculosis* are essential for meaningful pathogenesis studies using experimental infection, and in particular to allow comparisons between studies. However, even in very recent studies, accurate enumeration of infective doses has often received little attention, and doses have been recorded only as weight of organisms administered, or even as weight of infected mucosa. Chapter 2 of this thesis describes the development of techniques for the enumeration of ovine strains of *M. a. paratuberculosis* which will be helpful in further studies of both pathogenesis and epidemiology. The use of direct counts, spectrophotometry and plate counts were compared to most probable number (MPN) estimations, which until this study was the only technique available for the enumeration of viable ovine strain *M. a. paratuberculosis*. Doses of viable *M. a. paratuberculosis* given in the pen trial experiments described in Chapter 3 were based on direct counts, and were within a log of the intended dose rates. At the time, this was considered to be an excellent result. Subsequently however, efficient storage of ovine strains of *M. a. paratuberculosis* at -80 °C was demonstrated, and a simple technique for estimating the numbers of organisms inoculated into Bactec vials, using the number of days taken for the cumulative growth index to reach 1000 (CGI1000), was developed. These latter techniques will allow future experimental infections at defined dose levels to be done with more confidence and accuracy than was possible in the pen trials of the current study.

The efficient storage at -80 °C and the simple Bactec CGI-based enumeration techniques were also employed to quantify the effect of the decontamination procedures necessary for isolation
of *M. a. paratuberculosis* (ovine strain) from clinical samples. Together, these enumeration techniques will also allow assessment of levels of infection in tissues or faeces, which will facilitate further insights into the pathogenesis of the disease, and also assist epidemiological investigations, where the levels of faecal excretion or pasture contamination are important. In the current study, these techniques were used to quantify numbers of *M. a. paratuberculosis* isolated from the tissues of experimentally infected weaners (Chapter 3), and to assess the relative sensitivity of the routine EMAI protocol for culture from tissues and a modification using a centrifugation step. The total effect of the decontamination procedures was shown to result in a detection limit of about $10^3$ organisms per tissue sample or $10^2$ per faecal sample. The fact that detection limits are at least $10^2$ organisms is important for the interpretation of culture results. We need to avoid thinking that animals which are culture-negative for *M. a. paratuberculosis* are not infected. They may indeed be uninfected, but could also be infected at a level below the detection limit for the culture technique.

The field studies detailed in Chapter 4 suggested that age has little effect on the early establishment of infection, since neonates, weaners and ewes had similar infection levels after similar periods of exposure. These findings are consistent with earlier studies with bovine strains which demonstrated similar levels of tissue infection 2½ months post-infection in sheep infected from 3 weeks to 20 months of age. Published studies suggest that infection of older animals, however, is less likely to progress to clinical disease, but the short duration of the current work did not allow any such assessment. Also of interest was the finding of the current study that lambs born from an infected ewe flock became culture-positive sooner than naive lambs suckling uninfected ewes introduced to the same infected environment. This suggests the possibility of transmission of infection directly from infected ewes to their offspring. Congenital infection (reported frequently in cattle but only once in sheep), infection via the milk, particularly colostrum, or simply enhanced faecal-oral transmission (eg. by exposure to faecally contaminated udders) are all possible. This early infection of lambs born from an infected flock could be important in the understanding of pathogenesis and epidemiology of OJD, and may have significance for the design of disease control programs. It warrants further investigation.

A proposed mechanism for both the development of age-related immunity and the particular susceptibility of ruminants to Johne's disease is the unique anatomy and physiology of the ileal Peyer's patch (PP) in young ruminants. The earliest lesions of Johne's disease are usually seen
in the interfollicular areas of the PP of the terminal ileum (TI), and later the TI is the location of the most severe lesions, particularly in sheep. The ruminant ileal PP reaches its greatest development soon after birth and involutes from about 6 months of age. Anatomically it contains abundant lymphoid follicles (mainly B cells) with little parafollicular tissue (mainly T cells) and functionally it is the major site for B-cell development and production.\textsuperscript{248} The immunoperoxidase (IPX) findings described in Chapter 5 reinforce that the terminal ileal PP in the young ruminant is very different from the jejunal PP. In this study fewer CD4\(^+\), CD8\(^+\), \(\gamma\delta\) T cells and CD45R\(^+\) (mainly B) cells were present in the interfollicular zones of the ileal PP. T helper (CD4\(^+\)) cells and cytotoxic (CD8\(^+\)) T cells are central to the adaptive immune response in mycobacterial infection,\textsuperscript{298} while \(\gamma\delta\) T cells may be important in the innate immune response as well.\textsuperscript{341} B cells can act as antigen-presenting cells, and have been shown to have a role in resistance to mycobacterial infection in mice,\textsuperscript{353} and possibly in resistance to clinical paratuberculosis in cattle.\textsuperscript{354} The interfollicular areas of the ileal PP of the young ruminant are relatively deficient in all of these, which may provide a local environment permissive to the multiplication of \(M.\ a.\ paratuberculosis\) within macrophages. Older ruminants (and non-ruminant species of all ages) lack this permissive area. Even though the ileal PP normally involutes well before any clinical signs of Johne's disease are usually apparent, the presence of such a permissive area may be of significance to give \(M.\ a.\ paratuberculosis\) a foothold in the host. Once sufficient multiplication of mycobacteria has occurred, the mycobacteria themselves may depress the immune responses of the host. For example, induction of NO synthesis by \(M.\ avium\) or \(M.\ tuberculosi\)s infection of macrophages has been shown to cause decreased survival of CD4\(^+\)T cells, reduced granuloma formation and reduced IFN-\(\gamma\) levels in mice.\textsuperscript{122, 233} \(M.\ a.\ paratuberculosis\) has been shown to decrease MHC expression by macrophages,\textsuperscript{355} and some recent studies in cattle suggest that an alteration in function of chronically infected macrophages may induce apoptosis of reactive T cells leading to a progressive local depletion of Th1 cells.\textsuperscript{163} Other studies have shown that \(M.\ a.\ paratuberculosis\)-specific \(\gamma\delta\) T lymphocytes exhibit cytotoxic activity against antigen-primed CD4\(^+\)T cells.\textsuperscript{60} Down-regulation of CD1 expression on antigen presenting cells (APC) is another reported immune-evasion mechanism of mycobacteria,\textsuperscript{341} and in this study for the first time, a decrease in cells expressing CD1b was demonstrated in the mesenteric lymph nodes (MLN) of infected lambs. In an infected host, a gradual build-up of mycobacterial infection may eventually pass some critical point beyond which effective Th1 responses can no longer be maintained. Thus, early increases in numbers of mycobacteria in the permissive environment of the ileal PP may be critical to the pathogenesis of OJD.
Necropsy, both in the pen trial and field investigations, was timed to maximise the numbers of sheep from which the organism might be cultured. The results of a number of previous studies, when taken together suggest that there is a “window period”, several months after first infection, when many exposed sheep are likely to be positive by intestinal or MLN culture. *M. a. paratuberculosis* is rarely isolated from the tissues of infected sheep in the first few weeks to a month after infection, whereas cultures undertaken from one to several months post infection are more likely to be positive. In sheep exposed to low doses (equivalent to natural exposure?) the numbers of sheep positive on tissue culture then decline. This “window period” may correspond to the latter part of the classical stage 2 of tuberculosis during which logarithmic growth of mycobacteria occurs within macrophages which are not sufficiently activated to destroy them. Organisms may be inhibited by host innate immune responses soon after infection, and it may take a month or two before organisms surviving within macrophages increase sufficiently to be detected by culture (remember that a detection limit of $10^2$ to $10^3$ organisms was demonstrated in this study). Later (3 to 4 months post-infection), developing cell mediated immunity (CMI) may significantly limit and even reduce numbers of organisms in many sheep. In some sheep, infection will progress and larger numbers of organisms will be present, facilitating diagnosis in those particular sheep by culture or other means.

The factors influencing which infected sheep limit the multiplication of *M. a. paratuberculosis* and which sheep suffer progressive disease are central to the understanding of the pathogenesis and epidemiology of OJD and to developing control strategies for the disease on infected properties. These factors may include age at first infection and level of exposure (both discussed above), intercurrent stressors (eg poor diet, lactation, climatic stress, other infections, hormonal effects), and genetic resistance of the host animal.

The current research was not designed to examine possible intercurrent stressors, but the results do offer a few hints that such stressors may have an effect. In the field trials conducted on Farm H, Group Ewe-2L was remarkable in that 60% of ewes examined 8 months post-exposure were infected (and one had histological lesions), compared to only 10% of their lambs after an identical exposure history. While this difference may simply reflect the greater likelihood of grazing ewes to pick up an infectious dose off pasture as compared to their suckling lambs, it could also be that the stress of lactation reduced the ewes’ resistance to the
establishment of infection. This is consistent with literature reports describing an association of clinical Johne’s disease with reproductive stressors, such as disease in heifers at first parturition/lactation,\textsuperscript{132} and disease in lactating ewes.\textsuperscript{210}

The role of the host’s genome in the resistance to Johne’s disease has received little attention to date. Traditional selection of sheep for resistance is unlikely to be practical because of the chronic nature of Johne’s disease and the age at which disease usually manifests. However, if specific alleles of known genes which confer resistance to the development of Johne's disease could be identified, then rams possessing those alleles could be used in severely affected flocks in a cost-effective manner. Preliminary investigations in the current study did indicate possible associations of polymorphisms at the NRAMP and MHC loci with susceptibility to Johne's disease in sheep. These findings were not surprising, given recent evidence for genetic influences on other mycobacterial diseases in cattle and humans,\textsuperscript{202, 28, 29, 50, 19189} and findings from experimental \textit{M. a. paratuberculosis} infections in mice.\textsuperscript{104, 333, 346} The major problem for investigations into the genetics of paratuberculosis resistance in ruminants is the difficulty in accurately identifying susceptible or resistant animals. Due to the focal or segmental nature of Johne's disease pathology,\textsuperscript{64} the lack of consistent gross lesions (especially in sheep)\textsuperscript{51} and the limited amount of tissue that can practically be examined by histopathology, some lesions will be missed even during a thorough necropsy. The problems are compounded in the live animal, for which no test or combination of tests will unequivocally determine an animal's infection status. Moreover, simple infection with \textit{M. a. paratuberculosis} needs to be differentiated from actual disease. For example, tests for CMI such as the IFN-\( \gamma \) test have shown some promise in identifying infected sheep, especially in the early stages. However, some infected animals with the largest IFN-\( \gamma \) responses later had no evidence of clinical disease.\textsuperscript{131} If selection based on IFN-\( \gamma \) response were used to remove infected animals from a flock, many possibly very resistant sheep would be removed also. While serological tests for Johne's disease have low sensitivity, their specificity is high, and positive serology is usually associated with severe disease.\textsuperscript{256, 324} Because large numbers of individual sheep can be efficiently screened serologically, these tests might be useful for identifying at least some susceptible animals. Similar conclusions for the influence of some alleles in the current study were reached based on serology alone or on more detailed phenotypic assessment, suggesting that such an approach might reasonably be investigated in future studies.
To reiterate, the factors influencing which infected sheep limit the multiplication of *M. a. paratuberculosis* and which sheep suffer progressive disease are central to the understanding of the pathogenesis of OJD. In the field trials in this study, 60% of sheep in a continuously highly contaminated environment, examined 8 to 12 months after first exposure, were found to be infected, many more than would later succumb to OJD, suggesting that recovery or latent infection may be occurring in many infected sheep. Study of the immune responses, especially local responses, of sheep at this critical early stage will ultimately help to determine the mechanisms of resistance or susceptibility to progressive *M. a. paratuberculosis* infection. For example, some studies have shown that infected sheep with the highest IFN-γ or DTH responses (both systemic responses) may later have the least evidence of disease.$^{114,131}$ However, most research to date has concentrated on diseased animals, or on animals overwhelmed by artificially very high doses in experimental infection. The same has been true of much research into the mycobacterial diseases of humans. This was pointed out succinctly for leprosy by Cree and Smith recently,$^{80}$ commenting on their disillusionment with “the overconcentration of studies on patients, who have demonstrably failed immunologically by getting leprosy.” Once animals with *M. a. paratuberculosis* infection become severely diseased they too have demonstrably failed immunologically. The infected lambs in the current immunology study (Chapter 5) were just beginning to develop CMI as shown by their delayed type hypersensitivity (DTH) and IFN-γ responses. The timing was consistent with previous experimental work in sheep in which DTH was first seen 8 weeks after experimental infection,$^{156}$ and IFN-γ assays first returned positive results 9-18 weeks after infection.$^{130,314,318}$ But at this early stage there was no disease, and in this low-dose ovine strain study no lesions were present either. The immunological findings were, however, similar to those of the few other studies of early infection, despite these other studies using higher doses of the bovine strain.$^{26,24}$ CD4⁺, CD8⁺, and γδ-T cell numbers were all increased in intestinal PP and MLN of infected lambs. This suggests that, although not typical of natural infection, the immunological changes found in these other studies at these early stages may be representative of those occurring in natural infection.

The current studies also addressed the problems of early diagnosis of *M. a. paratuberculosis* infection. In both pen trials and in the field, culture of tissues was shown to be a sensitive method of early detection. In the pen trials, skin testing for DTH showed promise, identifying 66% of confirmed-infected lambs with 100% specificity. In the field, however, skin testing detected only 28% of infected sheep with 86% specificity. Nor were many of the sheep positive
by tissue culture detected by other ante-mortem tests for infection. IFN-γ testing detected only 10% with 97% specificity, and faecal culture detected only 12% with 100% specificity. The relatively low sensitivities for all these tests in this study reflect their use in the very early stage of the disease process. Histological lesions, often used as the “gold standard” for M. a. paratuberculosis infection in sheep, were also infrequent and were not found in sheep with less than 8 months of exposure. Only 17% of culture-positive sheep had typical histological lesions, although all sheep with such lesions were culture-positive. The cultural findings from this study are consistent with early reported studies in experimentally infected sheep,22 that culture of tissues is the most sensitive method currently available for the detection of early M. a. paratuberculosis infection. The current study is, in addition, the first demonstration of culture from tissues as a practical tool for the detection of early infection in flocks of sheep after natural exposure to ovine strains of M. a. paratuberculosis, and could have practical applications in assessing infectivity of pasture. Early detection by culture would be even more attractive if the indications from this study, that a single pooled tissue culture from each sheep was a reasonable alternative to multiple individual organ cultures, were confirmed in further trials. This might be investigated by abattoir slaughter and sampling of naive sheep of any age which have been running on suspect pastures for 6-12 months.

This study confirmed the reported predilection sites for early M. a. paratuberculosis infection as TI and MLN.26 150 241 In the pen trials five of six lambs were culture-positive in TI, ileocaecal valve and/or MLN, compared to a single lamb positive in the jejunum (this lamb was also positive in MLN and TI) and none in the duodenum, colon or caecum. In the field trials 42 of the 47 culture-positive sheep were detected by cultures of MLN and/or TI only. The question arises. Why does M. a. paratuberculosis localise in the intestinal PP and MLN, whereas other mycobacteria do not? It is probably a combination of both a particular adaptation of M. a. paratuberculosis and a weakness in the ruminant host defences in these areas. Rodent infections, for example, do localise, but not to the same extent, and except for immunocompromised strains of mice, much larger doses of M. a. paratuberculosis are needed to initiate infection.232 Although M. a. paratuberculosis has been shown to normally enter the host via the lymphoepithelial tissues of the gut,197 227 this is not the only reason for the observed localisation, since greatest levels of infection are seen in the same predilection sites even after experimental infections via novel routes.55 56 194
The positive histopathological findings from the present trial, although few, also provide some additional insight into the development of lesions in sheep. Most of the lesions were focal in distribution, with few or no AFB present – typical of the early lesions described by others. However, one 15 month old lamb, potentially exposed for 12 months, had lesions which were typical of the diffuse multibacillary form of OJD in only one of three ileal sections. Such pathology is usually associated with sheep having advanced disease, and is usually expected to be widespread in the intestine. The histopathological findings in this lamb suggest that in some sheep, focal lesions may progress directly to the diffuse multibacillary form, rather than gradually spread to be multifocal, and only later become multibacillary in character. The temporal changes in the pathology of OJD will only be resolved by detailed prospective studies in individual sheep, perhaps by repeated biopsy.

The current study has indicated a high level of infection in sheep in the first 6 to 12 months of natural exposure in a highly infected environment, and has also established techniques for consistent experimental infection which might be representative of natural exposure. Thus the course and outcome of *M. a. paratuberculosis* infection in individual sheep could be investigated using detailed prospective studies in reasonable numbers of sheep. In cattle, intestinal and MLN biopsy and subsequent culture has been shown to be a sensitive technique for early detection of infection, and a single report of biopsy after experimental infection of sheep suggests that the technique has promise in this species also. A preliminary study comparing the results of simulated TI and MLN biopsy with more detailed necropsy examination of lambs from an infected flock, found culture of biopsy samples to be about half as sensitive as the more detailed necropsy sampling in detecting early infection (Whittington and Reddacliff, unpublished). A sensitivity of 50% is well in excess of that demonstrated in field trials in this study for other antemortem tests at this early stage of infection (10%, 12% and 28% for IFN-γ, faecal culture and skin testing respectively). Thus culture of biopsy samples would be the most sensitive antemortem test for early infection in sheep. While biopsy might be too expensive and impractical as a routine diagnostic test, repeated biopsy studies from individual sheep may be useful experimental tools in the further study of the pathogenesis of OJD.
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