PATHOGENESIS OF TUBERCULOSIS

IN THE BRUSHTAIL POSSUM,

TRICHOSURUS VULPECULA

Hand-coloured steel engraving by W Jardine, 1843

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Massey University, Palmerston North, New Zealand

Michèle Mary Cooke

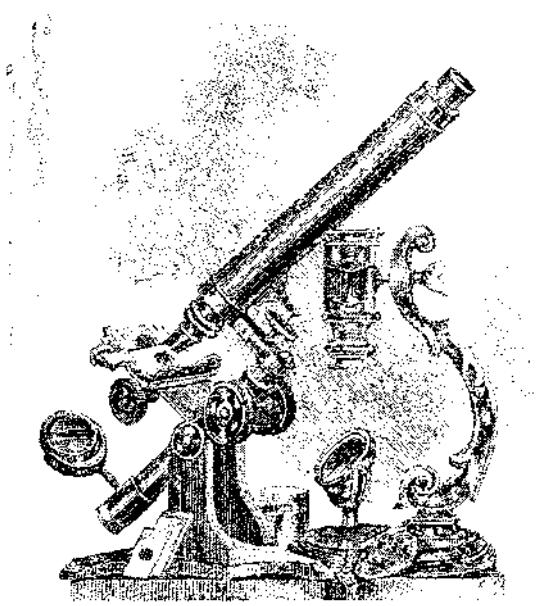
2000

"Too much reliance on the absence of macroscopic lesions has always constituted a source of error in pathological analysis."

Innes (1949)

"Not invisible but unnoticed, Watson. You did not know where to look, and so you missed all that was important".

Sherlock Holmes



Hogg J. The Microscope: its History, Construction, and Applications
Herbert Ingram & Co., London, 1855

ABSTRACT

The brushtail possum, *Trichosurus vulpecula*, is the main wildlife reservoir of *Mycobacterium bovis* infection for domestic species such as cattle and deer in New Zealand. Tuberculosis control and eradication are dependent on knowledge and understanding of the pathogenesis of the disease in this pest species, regardless of whether eradication or control of the disease by vaccination is contemplated.

Early field studies of tuberculosis in wild possums detected infected animals usually with advanced disease, and showed the two most common sites for macroscopic lesions were the respiratory tract and superficial lymph nodes. A comprehensive pathological study of the nature and distribution of lesions of naturally occurring tuberculosis which involved 117 non-terminally ill and 20 terminally ill possums was undertaken. Significantly more males (62%) than females (38%) were affected by the disease, and this is probably related to differences in behaviour. In non-terminally ill possums, the two most common sites for macroscopic lesions were the superficial lymph nodes (75%) and respiratory tract (69%). However, microscopic assessment of the distribution of total lesions disclosed 93% of lesions in superficial lymph nodes compared with 79% in the respiratory tract, indicating that the former are a predilection site for the establishment and development of lesions. This distribution raised the possibility that infection may occur via the percutaneous as well as the respiratory route. It was found that the disease disseminates early and rapidly via blood and lymph, and acid fast organisms increase in number in concert with increasing size and development of lesions.

In order to understand why lesions are common in the respiratory tract, a morphological study of the lung of the normal possum was undertaken. It revealed that the lung of the possum lacks a conventional mucociliary apparatus, a prime defence mechanism of the proximal airways against inhaled particles. However, this may be compensated for by the presence of Clara cells, which were abundant throughout the bronchial tree. Additionally, the lung appeared to be adequately supplied with mucosal associated lymphoid tissue. The lung of the possum may therefore be more susceptible to the deposition of particles larger than droplet nuclei into the airways than some other species.

Experimental respiratory infection with *M. bovis* involving the inoculation of 33 possums with 20-100 colony forming units (cfu) by the endo-bronchial route, and aerosol infection of 20 possums with 10-20 organisms, were completed over 4 and 5 week periods (respectively). This

allowed the study of the nature and development of pulmonary tuberculosis in possums, and comparison with the natural disease. Macroscopic lesions were largely confined to the respiratory tract, and at the microscopic level, there was a paucity of lesions in superficial lymph nodes, suggesting that in the natural disease percutaneous infection may be responsible for lesions in these nodes. A progression of lesion development from granulomatous through pyogranulomatous to large caseating lesions was observed. Rapid haematogenous and lymphatic spread occurred early in the experimentally induced disease. These findings confirm that the possum is highly susceptible to infection with *M. bovis*, and suggest that only an extremely small number of tubercle bacilli may be required to initiate the disease.

The results of experimental intra-dermal (I/D) inoculation of 5 x 10^6 cfu of BCG injected into the dorsal midline of the neck of 38 possums were followed over a 4 week period. This produced evidence that infection through the skin is associated with lesions in superficial lymph nodes. Although overall 76% of experimental possums had lesions in superficial nodes, few animals (21%) had lesions in the lower respiratory tract. The phenomenon of lesion resolution restricts the use of BCG to the study of early lesion development, however it avoids problems with overwhelming disease encountered in experiments using *M. bovis*. Further work using a very low dose of *M. bovis* via the percutaneous route will be necessary to understand whether the I/D route of infection operates simultaneously or sequentially with infection via the respiratory tract.

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LIST OF ABBREVIATIONS

AFOs	acid fast organisms
Anon.	Anonymous
BCG	Bacille Calmette-Guérin
°C	degrees centigrade/Celsius
cfu	colony forming units
cm	centimetre(s)
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
E/B	endo-bronchial
ed(s)	editor(s)
Edtn.	Edition
g	gram(s)
g	centrifugal force
G	gauge
GIT	gastrointestinal tract
h	hour(s)
H&E	haematoxylin and eosin
I/D	intra-dermal
I/M	intramuscular
I/N	intra-nasal
I/P	intra-peritoneal
I/T	intra-tracheal
I/V	intravenous
kg	kilogram(s)
L	litre(s)
lbs	pounds
LSD	Least Significant Difference test
MAFF	Ministry of Agriculture, Fisheries and Food
mg	milligram(s)
min.	minute(s)
mL	millilitre(s)
mm	millimetre(s)
No.	Number

NVL	no visible lesions
P	Page
PALS	periarteriolar lymphoid sheath(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pers. comm.	personal communication
pers. obs.	personal observation
p.i.	post-inoculation
Pp	Pages
S/C	subcutaneous
spp.	species
TEM	transmission electron microscopy
μm	micrometre(s) (micron(s))
UV	ultraviolet
ZN	Ziehl-Neelsen

CHAPTER 1. LITERATURE REVIEW

1.1 INTRODUCTION

Research efforts into the pathogenesis of tuberculosis have, up until recently, primarily focussed on the human disease and associated studies on laboratory animals. Before the advent of pasteurisation of milk, which was introduced into New Zealand in 1914, consumption of tuberculous milk resulted in disease in humans, particularly children, as well as in some other animal species (Figure 1.1). Although no more than 1% of tuberculous cattle excrete tubercle bacilli through milk, up to 100,000 acid fast organisms (AFOs) may be excreted per mL of milk (Feldman, 1941). The introduction of BCG vaccination and effective treatments of human tuberculosis saw a decline in work on the disease. As the eradication of tuberculosis in dairy cattle became a priority, the interest in disease caused by *Mycobacterium bovis* in animals intensified. At first, most work was concentrated on applied research aimed at national eradication programmes based upon test and slaughter regimes. The early success of eradication programmes in cattle has, in countries such as Britain, Ireland and New Zealand, given way to an appreciation that wild and feral animals infected with *M. bovis* constitute a significant threat to final eradication of the infection in domesticated animals.

Figure 1.1 Ingestion of unpasteurised milk from a tuberculous bovine udder was a common cause of tuberculosis in cats in Britain (Jennings, 1949). (Frank Lane Picture Agency, Acme Cards, London).

After a brief review of the historical aspects of tuberculosis, this review will concentrate on existing knowledge of the pathogenesis of *M. bovis* infection in mammals as a basis for the study of the infection in the Australian brushtail possum (*Trichosurus vulpecula*). This animal, which is thought to number between 60 and 70 million in New Zealand (Montague, 2000), is now recognised as the major reservoir host for *M. bovis* infection in the New Zealand environment (Julian, 1981), a rôle which is analogous to that played by the badger, *Meles meles*, in the British Isles.

1.2 THE HISTORY OF TUBERCULOSIS

1.2.1 Early history of the disease

Tuberculosis has been known to exist in humans and other mammals since at least classical times. It is likely it originated in Europe (Francis, 1958; Kiple, 1996), and the Romans identified tuberculosis in their farmed animals (Hunter, 1996). Tuberculosis was discovered in the mummified remains of a woman in the New World (Americas) dating back to approximately 900 AD (Toufexis, 1994).

Koch was the first to see the tubercle bacillus. In 1882, using a strong blue stain, he detected tubercle bacilli in lesions from a labourer who died of generalised tuberculosis. Later he was able to develop media capable of growing the bacteria which had been isolated from humans with "consumption" (de Kruif, 1927). Using his own postulates, he satisfied the four requirements viz. (1) the specific organism must be proven to be present in every instance of the infectious disease; (2) the organism must be capable of being cultivated in pure culture; (3) inoculating an experimental animal with the culture would reproduce the disease; and (4) the organisms could be recovered from the inoculated animal and grown again in a pure culture. His persistent experimentation resulted in the reproduction of the disease in other mammals, it provided proof of aerosol infection, and it demonstrated haematogenous spread. To quote from de Kruif's classical description of Koch's impressions: "What devils they are, those germs – from that one place in the guinea-pig's groin they have sneaked everywhere into his body, they have gnawed – they have grown through the walls of his arteries...the blood has carried them into his bones...into the farthest corner of his brain..."

It was not until 1896 that Lehmann and Neumann named the tubercle bacillus *Mycobacterium tuberculosis*. The "*M. tuberculosis* complex" is now known to consist of four species:

M. tuberculosis, *M. bovis*, *M. africanum* and *M. microti* (Cooper *et al.*, 1989), although *M. bovis* was not officially accorded separate species status until 1970 (Karlson and Lessel, 1970). In more recent times, diagnostic and epidemiological studies on tuberculosis of animals have been greatly facilitated by the advent of molecular technology. Techniques such as DNA fingerprinting, hybridisation with DNA probes and PCR-based typing methods have all provided new information on the nature and relatedness of tuberculosis isolates (Collins *et al.*, 1993; Aranaz *et al.*, 1998; Costello *et al.*, 1999). These techniques have proven to be particularly useful for characterising 'new' or atypical isolates that are not readily typable using traditional methods.

1.2.2 Bovine tuberculosis in New Zealand

Bovine tuberculosis was most probably introduced into New Zealand with the importation of cattle in the 1840's (Hickling, 1991). For public health reasons, voluntary testing of dairy cattle for tuberculosis was initiated in 1945, and became compulsory for all dairy herds in 1961. Compulsory testing of beef herds began in 1970 and of farmed deer in 1990 (O'Neil and Pharo, 1995). However, in some areas, normal test and slaughter schemes did not have the expected effects of disease eradication. The reason for this was the presence of infected possums, the major local wildlife reservoir for *M. bovis*. It therefore became apparent that unless infected possum populations could be significantly reduced and held at low levels, a test and slaughter policy alone would be considered insufficient for adequate control of bovine tuberculosis in New Zealand.

Prior to the discovery of the organism in brushtail possums in 1967 (Ekdahl *et al.*, 1970), tuberculosis was reported in wild red deer (*Cervus elaphus*) in 1956 (Livingstone, 1994), a hedgehog (*Erinaceus europaeus*) in 1957 (Brockie, 1990), and in feral pigs (*Sus scrofa*) in 1962 (Allen, 1991). The most recent mammalian species in which *M. bovis* has been discovered was a free-living hare (*Lepus europaeus occidentalis*) in 1992 (Cooke *et al.*, 1993). *Mycobacterium bovis* infection has now been recorded from most mammalian species in New Zealand, except wallabies (*Macropus, Petrogale* and *Wallabia* spp.), bats (*Mystacina tuberculata* and *Chalinolobus tuberculatus*), rats (*Rattus* spp.), mice (*Mus musculus*), pinnipeds, weasels (*Mustela nivalis*), chamois (*Rupicapra rupicapra*), thar (*Hemitragus jemlahicus*), four cervid species: sambar deer (*Cervus unicolor*), rusa deer (*Cervus timorensis*), white-tailed deer (*Odocoileus virginianus*), and moose (*Alces alces*), llamas (*Lama glama*), and alpacas (*L. pacos*).

1.3 *MYCOBACTERIUM BOVIS*

1.3.1 General characteristics of the organism

Mycobacterium bovis is a prokaryotic micro-organism belonging to the taxon Mycobacteriaceae, in the order Actinomycetales, a group of Gram positive bacteria. It is a non-motile, non-spore-forming pleomorphic rod-shaped bacillus with a lipid-rich cell wall, necessitating the use of hot concentrated carbol fuchsin, as in the Ziehl-Neelsen (ZN) stain, for visualisation. Once stained this way the bacilli are difficult to decolourise, even with acids, hence the term acid fast organisms. The organism is an intracellular parasite, capable of replication and multiplication in the cytoplasm of cells such as macrophages.

1.4 TRANSMISSION OF BOVINE TUBERCULOSIS

1.4.1 Introduction

The transmission of tuberculosis is inextricably linked with survival of the organism in the environment. Factors affecting the survival and/or multiplication of pathogenic organisms in the environment are the availability of nutrients, temperature, amount of organic matter present, sunlight/shade, moisture, pH, oxygen tension, inorganic ions and microbial flora (Wray, 1975). Protection of mycobacteria may be afforded by the presence of faecal material (Williams and Hoy, 1930). Survival of pathogenic bacteria on grassland may be affected by their location on the herbage or soil, or in the soil or sub-soil. This is because each location has differences in the availability of nutrients, temperature, moisture, pH, exposure to sunlight (ultra violet (UV) radiation), soil structure, dissolved oxygen, natural microflora and their interactions, presence of naturally occurring antibiotics in soil, and whether the bacteria are in a vegetative or spore form (Kelly and Collins, 1978).

Tanner and Michel (1999) emphasised that moist and shady conditions enhanced the survival of *M. bovis*, and survival of the organism is also enhanced if desiccation and evaporation are minimised. Mitscherlich and Marth (1984) found that *M. bovis* survived longer if shaded than in direct sunlight. Survival of *M. bovis* is longer in winter than in summer (Williams and Hoy, 1930; Maddock, 1934; MAFF, 1979; Nolan and Wilesmith 1994). King *et al.* (1999) demonstrated that the survival of *M. bovis* was favoured by low levels of UV radiation, low

temperatures, and high relative humidity, which are typically experienced in spring in England. However, there is a difference between the survival time of organisms and their remaining infectivity (Morris *et al.*, 1994), which is difficult to measure. The reasons for this proposed phenomenon are not clear.

Most of the work on the viability of *M. bovis* in the environment has been experimental, and conducted under unnatural conditions, resulting in survival times which are longer than in naturally infected material (Morris *et al.*, 1994). Additionally, many of these experiments have been conducted in England, where the climate and farming practices differ from those in New Zealand. It is probable that the survival rates of *M. bovis* in Britain are higher than those that occur under most natural conditions in New Zealand, because of differences in climate.

1.4.2 Routes of infection and shedding

Indications of the route of infection can be gained by studying the distribution of macroscopic lesions in the affected species. Infection via the respiratory tract typically results in a pattern of disease characterised by a combination of the initial site of infection and lesions in the regional lymph node (Dungworth, 1992). While it is a commonly accepted tenet that this pattern of tuberculosis indicates airborne infection, the situation is less clear when retropharyngeal lymph nodes or tonsils are affected. The former may indicate infection by either ingestion or inhalation, as the retropharyngeal lymph nodes receive afferent lymph from the floor of the mouth and pharynx and nasal cavity. Tonsils, on the other hand, have no afferent lymph vessels.

The disease in non-bovine ruminants and other herbivores, except for horses (*Equus caballus*), is essentially similar to that observed in cattle, in which tuberculosis is principally pulmonary (Francis, 1958), and may therefore generate infectious aerosols. On the other hand, carnivorous species, such as mustelids and cats (*Felis catus*), mainly attain infection via the oral route, with lesions most commonly occurring in mesenteric lymph nodes (Lepper and Corner, 1983).

The biological behaviour of each species also has a bearing on the projected routes of infection. This will involve the social interactions between animals of both the same and different species, and is often related to behaviour such as dominance, inquisitiveness, and gregariousness. For instance, Corner *et al.* (2000) found that disease transmission between possums in cage trials was more likely to occur in those animals which were highly socially interactive. Although this observation contrasts with the natural disease, where higher levels of tuberculosis are normally

found in immature possums, this latter phenomenon is most probably due to pseudo-vertical transmission, which is also closely associated with social behaviour in these animals.

RESPIRATORY INFECTION

Airborne infection occurs by spread of droplet nuclei and dust (Langmuir, 1961). Droplet nuclei are droplets measuring less than 10 μm in diameter, expelled from the nose or mouth, containing dissolved substances or solid matter, whereas particles 10 μm or larger are trapped on the mucociliary surface of the upper respiratory tract and bronchial tree, or are expelled to the pharynx and swallowed and digested. Droplet nuclei in the 0.5 to 3 μm diameter size range escape upper respiratory tract removal, and have the highest probability for deposition in a region of the respiratory tract susceptible to infection, such as the smaller bronchi (Sonkin, 1951; Middlebrook, 1961; Mullenax *et al.*, 1964). The nuclei of these droplets settle slowly and are carried long distances by light air currents (Wells, 1934). Wells and Stone (1934) suggested that differences in the viability of a micro-organism in air seemed to be consistent with the aetiology, epidemiology and pathology of air-borne infection. That is, the enhanced survival of certain respiratory organisms in air may indicate an adaptation of these organisms to this mode of transmission. As droplet nuclei are hygroscopic, small nuclei (approximately 1 μm in diameter) increase in size (diameter) as the air becomes saturated with moisture in distal airways and settle out in alveoli (Wells, 1955). In his summary of the distribution and deposition of inhaled particles in the respiratory tract Hatch (1961) emphasised that droplet nuclei contribute more significantly to air-borne infection than coarser dust-borne organisms. Among the most likely candidates for aerial spread were those diseases which were uniquely dependent upon deposition of inhaled particles in alveolar spaces or which were initiated by much smaller doses in the lungs than at other sites. For instance, mice, which are relatively highly resistant to high doses of *M. bovis* inoculated via subcutaneous (S/C) or intra-dermal routes, are susceptible to comparatively small numbers (approximately 100 bacilli) inhaled via aerosol (Glover, 1944).

Aerosols derived from bacillary suspensions containing isolated tubercle bacilli are about ten times more tuberculogenic than those containing larger bacillary groups. This is related to clumping of bacteria, as well as the overall increased size of a droplet due to the increased number of bacteria. Inhalation of about three viable, virulent tubercle bacilli in fine dispersion results in a single pulmonary focus in the rabbit (*Oryctolagus cuniculus cuniculus*) (Lurie *et al.*, 1950). Ratcliffe and Pallidino (1953) found that a single tubercle bacillus, which is of the order of 0.3 to 0.6 μm in diameter and 1 to 4 μm in length, deposited separately on alveolar surfaces of the lung in a droplet nucleus, is capable of initiating tuberculosis. Similarly, Middlebrook (1961) found that one in every three or four droplet nuclei bearing single tubercle bacilli inhaled by guinea pigs (*Cavia porcellus*) reached pulmonary spaces. Particles such as these, which are

deposited in distal, nonciliated portions of the lung, are quickly transported within macrophages to a ciliated area or to regional lymphoid tissue.

Eructation results in transportation of aerosolised micro-organisms from the rumen to the respiratory tract (Mullenax *et al.*, 1964). As 200-300 L of gas is eructated daily by a mature cow, it seems likely that considerable numbers of bacteria may enter the lung. Therefore, cattle may indirectly develop pulmonary tuberculosis following ingestion of tubercle bacilli. Particles in moist droplets are more likely to be deposited in the lung than are dry or unattached particles, and many such moisture droplets probably exist in eructated gas.

Aerosols may arise from expectorated sputum, and act as potential droplet nuclei, or they may be aspirated back into the lung, where they could set up another nidus of infection. In the United Kingdom, the survival of *M. bovis* from infected badger excretions, secretions, and carcases were assessed (MAFF, 1979). Bacilli from bronchial secretions containing 10,000 to 200,000 organisms/mL (Lloyd, 1976), were recovered in large numbers (700 organisms/mL) after 4 weeks on grass in winter, and in scant numbers after 10 weeks, but survived less than 1 week in summer. Additional work by Nolan and Wilesmith (1994) showed recovery of viable organisms from bronchial pus on pasture after 70 days in winter but less than 2 weeks in summer. In North America, Whipple and Palmer (2000a) demonstrated respiratory infection of calves with *M. bovis* via indirect contact with experimentally infected white-tailed deer through contaminated feed/fomites.

ALIMENTARY INFECTION

As in the respiratory tract, the alimentary tract has local defence mechanisms which resist infection. Ingested bacteria must survive passage through the stomach. The pH or acidity of gastric juices do not usually kill or inhibit tubercle bacilli because of their waxy cell walls. However, specific enzymes, the nature of which has not been determined (Schwarting, 1945), may seriously injure tubercle bacilli, especially if they have been exposed to gastric juices for at least 10 hours (Schwarting, 1948).

It is generally accepted that infective material coughed up in sputum and subsequently swallowed can cause infection of the lower ileum/ileocaecal area of the intestine, and also result in faecal contamination (Patel and Abrahams, 1989; Huchzermeyer *et al.*, 1994). However, it is not possible to differentiate the presence of tubercle bacilli in faeces due to the uneventful passage of bacilli ingested from the environment, from bacilli passed in faeces by a tuberculous animal. Infection of the liver and/or hepatic lymph nodes may occur from primary intestinal

tuberculosis, secondarily from swallowed tuberculous sputum, haematogenous spread (Stamp, 1944), or from congenital infection.

Bovine faeces have long been considered an important source of environmental contamination with tubercle bacilli, yet the number of organisms in faeces is difficult to ascertain. This is due to a number of factors, such as the presence of other organisms and the bulk of faecal material, and just as it is not possible to differentiate pathogenic from non-pathogenic mycobacteria, it is not possible to distinguish viable from non-viable bacilli. Hancox (1999) recently claimed that tuberculous cattle with macroscopically visible lesions can shed 38 x 10^6 bacilli/day/30 lbs of faeces, but this was not substantiated by experimental data.

Although it is widely accepted that thousands, often millions, of tubercle bacilli are required to establish infection via the oral route, supportive evidence is scant (White and Minett, 1941; Dannenberg, 1989). M'Fadyean (1910) reviewed experimental evidence which showed that 10 mg of infective material was necessary to infect calves via the oral route. It has been estimated that 1 mg wet weight of animal material contains approximately 2-10 x 10^7 tubercle bacilli (Brown, 1983). Chaussé (1913) found that 13 million tubercle bacilli would not always infect sheep (*Ovis aries*) by the oral route. Lurie (1930) crowded guinea pigs together with tuberculous cage-mates, producing extensive faecal contamination of cages, which resulted in intestinal tuberculosis, whereas pulmonary tuberculosis was established when faecal contamination was eliminated. When both routes of infection could operate, lesions were more prominent in alimentary sites, which Lurie believed indicated that the establishment of alimentary tuberculosis somehow inhibited the development of pulmonary tuberculosis.

Maddock (1933, 1934, 1936) used guinea-pigs to test the viability of *M. bovis* excreted in the dung from calves artificially infected with large numbers of organisms and from a naturally infected cow, as well as that on artificially infected grass. While S/C inoculations produced disease and death in guinea-pigs, oral inoculations required prolonged exposure to high levels of infection to accomplish disease. Similarly, calves grazing contaminated pasture became infected only after repeated infection of pasture (at very high levels) over a prolonged (4 month) period of time. These experiments demonstrated that for cattle, the duration of infectivity was much shorter than the period during which organisms could be recovered artificially, and emphasised the decreased susceptibility to infection via the oral route. Maddock (1936) concluded that the likelihood of infection via the oral route in cattle through grazing would be remote, with spread of the disease most likely occurring by animal-to-animal contact.

Reports from Northern Ireland have supported findings which suggest that cattle become infected with *M. bovis* from faeces of other tuberculous cattle, and this is associated with the method of storage and application of manure and slurry (Christiansen *et al.*, 1992; Griffin, 1992). Hahesy *et al.* (1992) recovered mixed bacterial contaminants of slurry up to 300 metres from its distribution source using conventional slurry spreading methods in windy conditions. Schellner (1959) showed that the infection of two cattle occurred one week after grazing pasture plots irrigated with 10^2-10^{12} *M. bovis*/mL. A slurry of tuberculous tissues in saline stored at 5-10°C yielded *M. bovis* after one year, but *M. bovis* survived only 18 days in pond water contaminated with tuberculous cattle organs (Mitscherlich and Marth, 1984). Under usual farming practices, situations such as these would be extremely unlikely to occur, and if they did, they would be unlikely to cause infection via the oral route. However, Collins (pers. comm.) mentioned that the tubercle bacillus becomes inactive in slurry or on pasture, and although it will not culture on artificial medium very well, it can still infect a live host. Thus the issue of direct or indirect infection from infected slurry remains unresolved.

Survival of *M. bovis* in soil is generally longer than on pasture, particularly if it is protected from soil insects, (burrowing) animals and birds (Maddock, 1933; 1934), and appears to be longer with its increasing depth in the soil (Genov, 1965). Compared with experiments conducted in Europe, recovery of *M. bovis* from artificially infected soil in the warmer climes of Australia (Duffield and Young, 1985) and South Africa (Tanner and Michel, 1999) was shorter and negatively related to sunlight and temperature. Thus, despite grazing cattle ingesting up to 450 kg of soil per year (Healy, 1968), accumulations of bacilli in soil are unlikely to pose a great risk of infection to them.

Although tubercle bacilli might survive in undisturbed dung pads during the spelling of paddocks for 4-6 weeks prior to grazing under a rotational grazing system, one would expect the risk of infection via the oral route unlikely to increase. Mitscherlich and Marth (1984) reported that pastures on which tuberculous cattle were kept remained infective for healthy cattle for 7 days after their removal, whereas Jackson *et al.* (1995c) believe that contaminated pasture, particularly in summer, may be relatively unimportant in the epidemiology of tuberculosis in cattle, deer and possums. Francis (1947) pointed out that young cattle grazing heavily infected pasture, or grazing with heavily infected stock, have a low incidence of tuberculosis until they enter the cowshed.

Whipple and Palmer (2000b) determined the survival of *M. bovis* on feeds used for baiting white-tailed deer and isolated the organism from various feeds stored at 8°C for at least 16 weeks, and at 22°C for 7 days. Although these feeds could act as a source of infection for

other animals, infection via the oral route would probably be a rare event, due to the number of viable organisms required to initiate disease by this route.

INFECTION VIA THE INTEGUMENT

Mycobacterium bovis infection has been acquired via the integument in cats, ferrets (*Mustela furo*) and badgers (Tables 1.1 and 1.2). In cattle, Thoen and Himes (1986) stated that cervical lymph node lesions are evidence of infection through skin abrasions, since afferent lymph vessels are primarily from skin over the neck and caudally to rib 12, and over the forelimbs. M'Fadyean (1901) explained the presence of lesions in peripheral lymph nodes in experimental intravenously infected cattle by the escape of a trace of inoculum into the associated connective tissue during withdrawal of the needle from the vein. He noted that the affected nodes were on the same side as the site of inoculation, and because lesions were absent in lymph nodes on the opposite side of the animal, he believed that peripheral lymph node involvement was not an indication of generalisation of the disease. Brown (1983) found that the bulk of a S/C inoculation in rabbits localised at the injection site and some drained to the regional lymph tissue.

Glover (1944) calculated that the minimum infective dose of *M. bovis* for a guinea pig via the S/C route is approximately 10 bacilli. The experimental studies on possums by Corner and Presidente (1980, 1981) and Buddle *et al.* (1994), demonstrated the establishment of skin infection, and in the case of the latter work it was achieved with only small numbers of bacilli. DNA restriction analysis of a cluster of *M. bovis* skin infections in cats in the Hutt Valley, New Zealand, produced strong evidence that the source of infection most probably originated from a three-clinic veterinary practice in the region (de Lisle *et al.*, 1990). The site of the skin wounds, especially in the shoulder region, implies infection may have involved the inoculation of a small number of tubercle bacilli during either routine vaccinations or antibiotic injections.

The distribution of macroscopic lesions, and the likely routes of infection and shedding that have been recorded in *M. bovis* infection in species of mammal present in New Zealand, and overseas reports in mammals either not present in New Zealand or which have not been recorded in this country are presented in Tables 1.1 and 1.2 (respectively).

Table 1.1 Patterns of disease seen with *Mycobacterium bovis* infection in species of mammal present in New Zealand

Species	Macroscopic lesion sites	Route(s) of infection	Route(s) of shedding
ARTIODACTYLA:			
Bovidae:			
Cattle (*Bos taurus*)	Lung[1-15], retro ln[1-12], mesen ln[3,4,6,8,9,11-14,16]	Resp[3-6,9,11-13,15,17-19], oral[4,12,19]	*Aerosol[5,6,8,16,17,20]
Goat (*Capra hircus*)	Lung[21-26], head ln[22]	Resp[22,23]	Aerosol[25]
Sheep (*Ovis aries*)	Lung[27-33], mesen ln[27-29], liver[28,32]	Oral[27,28,32], resp[27]	Aerosol[31]
Cervidae:			
Fallow deer (*Dama dama*)	Head ln[34-38], lung[34,35,37-40], mesen ln[35-40]	Resp[34,37,38], oral[37,38]	Disch[37], aerosol[39]
Red deer (*Cervus elaphus elaphus*)	Retro ln[34,40-55], mesen ln[40,41,43,47-54,56], lung[34,41-45,47-58]	Oral[51,52,54,55], resp[34,48,51,52,54,55]	Disch[45,51,59], saliva[45,55,59], aerosol[45,52,54,59], faeces[45,54,59]
Sika deer (*Cervus nippon*)	Lung[60-62], retro ln[60,62], GIT[61]		Aerosol[61]
Wapiti (*Cervus elaphus nelsoni*)	Lung[63-67], retro ln[64-67], mesen ln[64-67]	Resp[66]	Aerosol[63,64,66,67]
Suidae:			
Pig (*Sus scrofa*)	Head ln[68,69-76], mesen ln[70-73,75,77], lung[74,77]	Oral[1,69-73,78]	Aerosol[74]
CARNIVORA:			
Canidae:			
Dog (*Canis familiaris*)	Liver[79-81], mesen ln[1,79,80,82], Lung[79-83]	Oral[1,80,82,84], resp[82,84]	Urine[80]
Felidae:			
Cat (*Felis catus*)	Skin[1,82,85-90], mesen ln[1,68,82,85-91], submandibular ln[68,86,89-92]	Oral[1,26,82-86,88], wounds[82,85,88]	Aerosol[26,82,93], disch[68,92-94]
Mustelidae:			
Ferret (*Mustela furo*)	Mesen ln[95-100], retro ln[95-97], pre-scap ln[95,101]	Oral[95,97,99,102-104], wounds[95,103]	Disch[95,97,103], saliva[101,103], milk[103]
Stoat (*Mustela erminea*)	Mesen ln[105]	Oral[105]	
INSECTIVORA:			
Erinaceidae:			
Hedgehog (*Erinaceus europaeus*)	Lung[106-108]	Oral[107], resp[105]	
MARSUPIALIA:			
Phalangeridae:			
Brushtail possum (*Trichosurus vulpecula*)	Lung, peripheral ln[a]	Resp[109-113], pseudo-vert[109-111], percut[109,110,112-114], oral[109,110]	Aerosol[111+2,115+6] disch[109-112,115,116] saliva, faeces, urine[112]
PERISSODACTYLA:			
Equidae:			
Horse (*Equus caballus*)	Mesen ln[21,78,117-121], spleen[78,117-121], liver[21,117,120]	Oral[118,119,122]	
RODENTIA:			
Leporidae:			
Hare (*Lepus europaeus occidentalis*)	Lung[105,123,124], mesen ln[124]	Resp?[105]	
Rabbit (*Oryctolagus cuniculus cuniculus*)	Lung, kidney[125,126], pre-scap ln[126]	Oral[125]	

For reasons of space, references can be found in Appendix IV.
retro = retropharyngeal; ln = lymph node; resp = respiratory; mesen = mesenteric; disch = discharging (wounds/sinuses); GIT = gastrointestinal; pre-scap = prescapular; pseudo-vert = pseudo-vertical; percut = percutaneous
*Aerosol excretion in cattle also includes nasal mucus
[a]See Table 1.3 for full details

Table 1.2 Overseas reports of features of *Mycobacterium bovis* infection occurring in mammals either not present in New Zealand or which have not been recorded in this country

Species	Macroscopic lesion sites	Route(s) of infection	Route(s) of shedding
ARTIODACTYLA:			
Bovidae:			
Antelopes:			
Antelope (*Oryx beisa*)	Lung[1]		Aerosol, faeces[1]
Arabian oryx (*Oryx leucoryx*)	Lung[1,2], mesen ln[2]	Resp[2]	Aerosol, faeces, urine[2]
Gemsbok (*Oryx gazella*)	Lung[1]		
Greater kudu (*Tragelaphus strepsiceros*)	Lung[3,4], spleen[3], subparotid ln[4,5]	Resp, oral[6,7], skin[7]	Disch[6]
Kafue lechwe (*Kobus leche kafuensis*)	Lung, mesen ln[8]	Resp[8]	Aerosol, faeces[8]
Springbok (*Antidorcas marsupialis*)	Lung[9]		
White-bearded gnu (*Connochaetes albo-jubatus*)	Lung[1]		
White-tailed gnu (*Connochaetes gnou*)	Lung[10]		
Bovine:			
American bison (*Bison bison*)	Head ln[11,12], resp tract[11-13]	Resp, oral[11]	
Buffalo (*Syncerus caffer*)	Resp tract[14-19]	Resp[6, 14,15,17,18]	Aerosol[14,15,17]
European bison (*Bison bonasus*)	Lung, mesen ln[20]		
Hiriana cattle	Mediastinal ln, bronchial ln[21]		
Water buffalo (*Bubalus bubalis*)	Resp tract[22-26]	Resp[24,26]	Aerosol[22]
Zebu cattle (*Bos indicus*)	Retro ln, resp tract[27]	Resp, skin[27]	Aerosol[27]
Caprine:			
Chamois (*Rupicapra rupicapra*)	Lung[28,29]		
Ovine:			
Burrhel sheep (*Pseudovis nahura*)	Lung[1]		
Camelidae:			
Camel (*Camelus* spp.)	Lung[30-33], liver[31], kidney[33]	Resp[31]	Urine[33]
Cervidae:			
Axis (chital) deer (*Axis axis*)	Lung[1,34]		
Mule deer (*Odocoileus hemionus*)	Bronchial ln, head ln[35]		
Reindeer (*Rangifer tarandus*)	Lung[1,10]		
Roe deer (*Capreolus capreolus*)	Lung[29,36], mesen ln[29]		
White-tailed deer (*Odocoileus virginianus*)	Lung[36-39], retro ln[36]	Resp, oral[36]	Aerosol[36]
Suidae:			
Warthog (*Phacochoerus aethiopicus*)	Submaxillary ln[40]	Oral[40]	
CARNIVORA:			
Canidae:			
Fennec fox (*Fennecus zerda*)	Liver, kidney, lung[41]		Urine[41]
Fox (*Vulpes vulpes*)	Mesen ln[42]	Oral[43,44]	Disch[43]
Wolf (*Canis lupus*)	Mesen ln[45]		
Felidae:			
Amur leopard (*Panthera pardus*)	Lung[46]	Oral?[46]	
Cheetah (*Acinonyx jubatus*)	Resp tract[47-49]	Oral[6,47]	Aerosol[47]
Lion (*Panthera leo*)	GIT[6], bone, eye[7], lung[6,47,48]	Oral[6,47], resp[47,50]	Aerosol[47,48,50]
Snow leopard (*Panthera uncia*)	Lung[46]	Oral?[46]	
Mustelidae:			
Badger (*Meles meles*)	Lung[51-63], kidney[51,52,54-56,58-62], head ln[55,56]	Resp[53-58,61,63-67], wounds[53-57,61-67], oral[53,56,68], pseudo-vert[53,55,56]	Urine[51-58,64,66,69-71], faeces[52-59,66,67,69-71], disch[51-55,58,66,69-71], aerosol[53-56,58,59,64,67,69-71], sputum[51,53-57,66,69,71]
Mink (*Mustela vison*)	Mesen ln[42,72], retro ln[73]	Oral[43,44,72]	Disch[43]
Otariidae:			
Sealion (*Otaria bryonia*)	Lung[46]	Oral?[46]	
Procyonidae:			
Coati (*Nasua narica*)	Peritoneal serosa[1]		
Ursidae:			
Sun bear (*Helarctus malayanus*)	Lung, mesen ln[20]		
Viverridae:			
Pardine genet (*Genetta pardina*)	Chest[1]		
CHIROPTERA:			
Fruit bat (*Pteropus giganteus*)	Peritoneum, mesen ln[20]		
Indian fruit bat (*Pteropus medius*)	Lung[1]	Resp[1]	
MARSUPIALIA:			
Macropodidae:			
Rat kangaroo (*Aepyprymnus rufescens*)	Lung[1]		
PERISSODACTYLA:			
Rhinocerotidae:			
African rhinoceros (*Rhinoceros bicornis*)	Lung[1]		
Black rhinoceros (*Diceros bicornis*)	Lung, liver[74]		
Southern white rhinoceros (*Ceratotherium simum simum*)	Lung[75]		
Tapiridae:			
Malay tapir (*Tapirus indicus*)	Lung[10]		
PROBOSCIDEA:			
Elephantidae:			
African elephant (*Loxodonta africana*)	Peritoneal cav, mesen ln, lung[40]		
RODENTIA:			
Brown rat (*Rattus norvegicus*)	Intestine[28]	Oral[28]	
Porcupine (*Hystrix* spp.)	Lung[76]	Resp[76]	

For reasons of space, references can be found in Appendix V.
mesen = mesenteric; ln = lymph node; resp = respiratory; disch = discharging (sinuses/wounds); retro = retropharyngeal; GIT = gastrointestinal tract; pseudo-vert = pseudo-vertical; cav = cavity.

1.4.3 Modes of transmission in selected species

CATTLE IN NEW ZEALAND

Because the principal route of infection for cattle is the respiratory route, with the oral route of lesser importance, lesions are most commonly seen in the respiratory tract and retropharyngeal lymph nodes. Spread of infection from possums to cattle occurs relatively easily, but only rarely in the reverse direction (Collins *et al.*, 1988). Contact between possums and cattle probably occurs because of the natural curiosity of the latter and their tendency to investigate terminally ill possums, which may behave abnormally by wandering aimlessly in daylight (Morris and Pfeiffer, 1995) (Figure 1.2). As with deer (*Cervus* spp.), it is usually those animals high in the dominance hierarchy which are most inquisitive, and therefore most likely to contract tuberculosis (Morris and Pfeiffer, 1995). Conversely, sheep are less inquisitive than cattle and are very cautious about approaching different species (Morris and Pfeiffer, 1995). It is this behaviour, together with husbandry practices (Cordes *et al.*, 1981), which is likely to be why the disease is rarely reported in sheep kept outdoors, although sheep are considered highly susceptible to experimental infection with *M. bovis*.

Figure 1.2 Investigatory sniffing of a dazed possum by a heifer.

The main mode of cattle-to-cattle transmission is by aerosol inhalation. McIlroy *et al.* (1986) believed that all tuberculous cattle with lesions in lymph nodes associated with the respiratory system should be considered possible producers of infected aerosols and thus important sources of infection for other cattle both within and between herds. Infection of retropharyngeal lymph nodes may occur via either the respiratory or oral routes, but because of their common

association with respiratory tract lesions, the majority are thought to be due to inhalation (Stamp and Wilson, 1946).

CATTLE IN GREAT BRITAIN

As yet, no work has been undertaken to answer the question of whether an infective dose of *M. bovis* may be inhaled into the respiratory system during olfactory investigation, but Benham and Broom (1991) offered a suggestion from their behavioural studies of cattle. Cattle grazing pasture contaminated with badger excreta might create an aerosol when grazing the tops of grasses laden with heavy dew. Contaminated herbage was more commonly ingested when cattle were forced to be less selective due to limited supplies of attractive food. On the other hand, Hutchings *et al.* (1999) found that reducing badger population densities did not produce commensurate reductions in disease transmission risks from badgers to cattle, as the population reduction altered the badgers' scent-marking strategy, leading to subsequent changes in investigative and grazing behaviour of cattle. Badgers placed a greater proportion of urinations at crossing points rather than at latrines. Crossing point urinations are not avoided by cattle, whereas latrines are. These authors believed that olfactory investigation of urine-contaminated pasture by cattle was far more likely to create explosive aerosolised inhalation than was faecal contamination.

CERVIDAE

The high prevalence of tuberculosis in feral deer in densely forested areas of New Zealand suggests that deer-to-deer and/or possum-to-deer transmission must occur in order to maintain the disease in deer (Nugent and Lugton, 1995). Sauter and Morris (1995b) demonstrated that, as with cattle, deer high in the dominance hierarchy may investigate tuberculous possums, and thus risk becoming infected with *M. bovis*. Lugton *et al.* (1998) believed that deer-to-deer transmission is density-dependent and that the threshold for disease "retention" between deer is at or above the densities at which deer routinely occur in the wild in New Zealand. Deer-to-cattle transmission appears a rare event, although infected farmed or dispersing feral deer are thought to be the principle initiator of entirely new areas of wildlife infection, apparently via scavengers feeding on infected deer carcases (Morris and Pfeiffer, 1995). Deer-to-possum transmission may occur when possums feed on tuberculous deer carcases (Nugent *et al.*, 2000a).

A study by de Lisle and Havill (1985), found 75% of tuberculous feral deer had lesions in the thorax, which contrasted with the situation in farmed deer where medial retropharyngeal lymph nodes were the main predilection site for macroscopic lesions (Hathaway *et al.*, 1994). Lugton *et al.* (1998) took the view that the differences were most probably due to the fact that farmed deer occur at high densities and there are more opportunities for contact, including that

associated with agonistic behaviour. However, the difference may also be related to the fact that farmed deer are under compulsory tuberculosis testing, resulting in early detection of the disease.

Lugton *et al.* (1998) suggested that because *M. bovis* was cultured from the oropharyngeal tonsil of nearly two thirds of infected deer in their study, acquisition of infection of the tonsil may be one of the more important routes of natural infection in deer, after which there is subsequent rapid haematogenous spread to the lungs. Experimental tonsillar inoculation of white-tailed deer with *M. bovis* by Palmer *et al.* (1999a) resulted in infection of retropharyngeal lymph nodes. These authors proposed that mycobacteria present in the oropharynx or mouth, through inhalation or ingestion, would be processed by the tonsil and likely carried through the lymphatics to retropharyngeal lymph nodes.

Shedding of tubercle bacilli from tuberculous deer may occur via discharging sinuses from infected lymph nodes of the head and neck. Although few bacilli are shed by the majority of infected deer, experimental infection of white-tailed deer with *M. bovis* revealed that severe disseminated tuberculosis was not a prerequisite for the presence of bacteria in saliva or nasal secretions (Palmer *et al.*, 1999a).

ANTELOPE SPECIES

Kudu (*Tragelaphus strepsiceros*) may become infected via the cutaneous route, with lesions commonly presenting in parotid lymph nodes (Table 1.2). Thorburn and Thomas (1940) suggested this might be due to scarification of skin around the ears during scratching at ticks and horn flies with hind hooves contaminated with *M. bovis* in environments densely populated with tuberculous cattle. Bengis (1999) provided another theory. He suggested that because kudu have very large ears, which are relatively smooth and hairless on the inside of the pinna, they may become scratched while browsing thorn trees. Exudate from fistulating abscessed parotid lymph nodes of infected kudu browsing at the same level may contaminate these breaks in the epidermis.

MUSTELIDS

Ferrets

Since ferrets are scavenging carnivores it is not surprising that the majority of macroscopic tuberculous lesions are in mesenteric (jejunal) lymph nodes, followed thereafter by retropharyngeal lymph nodes (Ragg *et al.*, 1995c; Lugton *et al.*, 1997b). This is consistent with infection via the oral route. However, the presence of approximately 20% of lesions in peripheral lymph nodes, has led to the suggestion that these originate via percutaneous infection

from intraspecific bites (Ragg *et al.*, 1995c; Lugton *et al.*, 1997c). Respiratory infection is uncommon (Ragg *et al.*, 1995a; Lugton *et al.*, 1997b).

The discovery of *M. bovis* infection in ferrets in certain areas of New Zealand, such as Otago and the Mackenzie Basin, has raised the possibility that this species may also act as a reservoir host of infection (Walker *et al.*, 1993; Ragg *et al.*, 1995b). These areas have a high density of ferrets with a high prevalence of *M. bovis* infection, whereas both the numbers of possums and the prevalence of tuberculous possums are low. Extensive surveys by Caley (1998) have produced convincing evidence supporting the hypothesis that tuberculous possums are the major underlying source of *M. bovis* infection for feral ferrets. However, the experimental work of Qureshi *et al.* (2000) showed that ferret-to-ferret transmission of tuberculosis could occur in some situations, and raised the possibility that horizontal transmission might also occur in high density feral populations.

There is evidence that about a quarter of tuberculous ferrets shed bacilli orally. Although aerosol shedding is likely to be rare, it may occur in animals with advanced generalised disease. A similar situation is probable with regard to faecal and urinary shedding. Since the organism has been isolated from one of eight sets of mammary glands, infection of milk from affected glands could transmit disease to suckling kittens (Lugton *et al.*, 1997c).

Badgers

A different lesion pattern to that of many carnivores is seen in the badger, the primary wildlife reservoir of *M. bovis* in the United Kingdom. In this species, the major lesion site is also the lung, reflecting infection via the respiratory route, but the kidney is a common predilection site for secondary lesions (Gallagher *et al.*, 1976). This results in urine containing large numbers of bacilli (up to 300,000 per mL) (MAFF, 1979) and represents a source of infection for cattle grazing contaminated pasture (Hutchings and Harris, 1999). Lesions in lymph nodes of the head of badgers have been attributed to wounds inflicted during intra-specific conflict. Gallagher and Nelson (1979) speculated that the mechanism of injection of tubercle bacilli into such wounds was from tuberculous lung discharges contaminating a badger's teeth. Clifton-Hadley *et al.* (1993) on the other hand suggested that intracellular *M. bovis* may preferentially be disseminated to sites of traumatic wounds or the lymph nodes which drain them. However, recent observations (Cooke, 2000) of infected badgers have shown that saliva may also be a source of bacilli for bite wound infection, as indicated by lesions found in salivary glands (Figure 1.3).

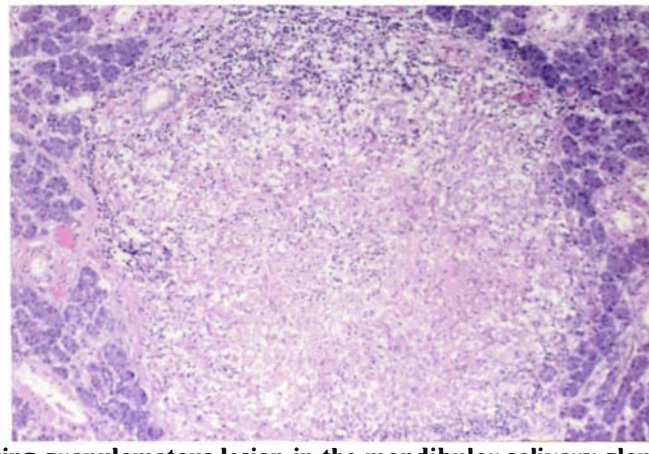

Figure 1.3 A necrotising granulomatous lesion in the mandibular salivary gland of a tuberculous badger. The lesion contained several acid fast organisms. H&E. Magnification = 40x.

FELIDAE

Unexpectedly, tuberculosis in the obligate carnivores, lions (*Panthera leo*) and cheetahs (*Acinonyx jubatus*), has been recorded in the lung, suggesting infection via the respiratory tract (Table 1.2). Bengis (1999) has put forward two possible theories. Large predators frequently suffocate their prey, either by biting through and occluding the larynx/trachea, or by biting over the muzzle to occlude the mouth and nostrils. During its agonal gasping, a prey animal with open pulmonary tuberculosis could thus infect the predator. Alternatively, normal activities, such as panting, grooming, or feeding, between members of a pride, would allow direct intraspecific horizontal aerosol transmission. This latter theory would suggest that the disease is self-maintaining within a pride, implying that lions are reservoir hosts. However, this aspect of disease transmission was not discussed.

OTHER SPECIES IN NEW ZEALAND

Pigs

Seventy-seven of 251 (31%) feral pigs killed in Central Otago had histopathological evidence of tuberculosis (Wakelin and Churchman, 1991). Of these, 96% had involvement of lymph nodes of the head, and 33% had pulmonary lesions, denoting infection is probably most common via the oral route. Given that pigs are omnivores, the source of infection would most likely be scavenged carrion. The disease is considered self-limiting and transmission to other animals would be unlikely (de Lisle, 1994; McInerney *et al.*, 1995). However, the tuberculous carcases of pigs, cats and goats (*Capra hircus*) could provide a source of infection for scavenging species such as ferrets (Morris and Pfeiffer, 1995). Recent ongoing research indicates that such transmission occurs readily and makes pigs very useful 'sentinels' of *M. bovis* infection in possum or deer populations (Nugent *et al.*, 2000b).

Cats

A study of 76 tuberculous cats in New Zealand revealed nearly half had skin lesions, possibly as the result of wounds inflicted by tuberculous possums (de Lisle, 1993). In domestic cats, lymph node lesions were most commonly recorded in submandibular and mesenteric nodes, indicating infection via the oral route (de Lisle *et al.*, 1990), which in feral cats in New Zealand is most likely through scavenging tuberculous carcases.

Hedgehogs

Of interest is the finding by Lugton *et al.* (1995) that three of four tuberculous hedgehogs had lesions confined to the lung (the fourth had generalised tuberculosis, including extensive pulmonary involvement). These authors attributed this type of infection to the scavenging behaviour of these omnivorous animals, and suggested that uptake and replication of bacilli in macrophages, following initial phagocytosis from the gastrointestinal mucosal surface, resulted in entry of the bacilli into the circulation and subsequent infection of the lung, a predilection site for lesion development in *M. bovis* infection. This method of pathogenesis was originally advanced by von Behring (1903) who proposed that tuberculosis of broncho-mediastinal lymph nodes in cattle originated from tuberculosis of intestinal origin, produced by tubercle bacilli entering the body through the mucous membranes of the gastrointestinal tract (GIT). The bacteria were said to arrive at these nodes from the intestinal mucosa via the mesenteric lymph nodes, the thoracic duct, the bloodstream, the lungs and finally the bronchial lymph nodes. Although this circuitous route of development could be possible in theory, an aerosol route of entry in these cases is more probable. Reeve (1994) has suggested it is likely that the prey of a hedgehog is often detected by its odour. He also cited Herter (1965) who noted that hedgehogs spend a long time sniffing over dead, motionless, or very inactive food, whereas mobile prey is quickly seized. Conversely, Dimelow (1963) found that hedgehogs did not eat any dead or moribund prey.

1.5 THE PATHOLOGY OF TUBERCULOSIS IN MAMMALS

1.5.1 The pathology of tuberculosis in mammals in New Zealand

Among the wild and feral mammals in New Zealand affected with *M. bovis*, the disease is most common in possums and deer, which are likely to be responsible for infection of many other species (Cooke *et al.*, 1999a). As early detection is rare, animals under feral conditions usually have advanced disease as compared with that seen in infected animals in a domesticated state.

Many of the classical histopathological features which typify tuberculosis in cattle are not observed, or seen to a lesser extent, in other mammals. Generally, the histological lesions seen in wildlife in New Zealand are more florid and less discrete than the classical tuberculous granulomas. Mineralisation and fibrosis are rarely observed microscopically in lesions in the majority of mammals apart from cattle.

CATTLE

As with grazing quadripeds such as deer (Rhyan *et al.*, 1992), sheep (Cordes *et al.*, 1981; Davidson *et al.*, 1981), and kafue lechwe (*Kobus leche kafuensis*), a species of antelope (Gallagher *et al.*, 1972), there is a propensity for pulmonary lesions in cattle to occur in peripheral dorsocaudal areas of the lung (Medlar, 1940; Stamp, 1948). In cattle, superficial lesions are more common than lesions deep in the pulmonary parenchyma, even allowing for differences in volume between lung lobes (Medlar, 1940). McIlroy *et al.* (1986) found 90% of all pulmonary lesions in the diaphragmatic (caudal) lobes, nearly half of which occurred in the distal one third. This distribution has been attributed to the normal standing posture of cattle, whereby the most elevated, dorsocaudal, portion of the bovine lung is the most common site of infection. Pulmonary tuberculosis in humans, in which lesions occur mainly in the dorsocephalic (apical) regions (Patel and Abrahams, 1989), is similarly in the most elevated part of the lung.

The macroscopic appearance of lesions may range from those of a small, firm, greyish or yellowish-white nodule to a definitive, creamy-white tubercle with a gritty centre (Francis, 1958). Like the lesions of *M. tuberculosis* infection in humans, the microscopic appearance of *M. bovis* infection in cattle is characterised by central caseation and mineralisation, with giant cells located around the periphery of this central area, merging into a collection of epithelioid cells, macrophages and lymphocytes, and pulmonary lesions are often walled off peripherally by fibrous tissue. Acid fast organisms are usually few in number, often requiring careful scrutiny of several high-powered fields for location and identification.

Intra-pulmonary dissemination occurs via the bronchial passages, and secondary lesions, such as caseous lymphadenitis of mesenteric lymph nodes, arise from swallowing coughed up tuberculous exudate. Stamp (1944) believed that tuberculous pleurisy may result from either direct lymphatic drainage from a pulmonary lesion or via haematogenous spread.

Tuberculous peritonitis occurs late in the disease, and in the cow may lead to infection of uterine tubules to the placenta and thence to the foetus. Prior to tuberculosis control and

eradication schemes, the lesions seen in cattle were often advanced, with the result that uterine and mammary infections were frequently recorded.

Lesions present in hepatic and mesenteric lymph nodes and the intestine in the post-natal calf could be interpreted as post-natal infection via the oral route. However, Stamp and Wilson (1946) have provided evidence that this phenomenon is more likely to arise following sublethal infection *in utero* along the umbilical vein. Pulmonary tuberculosis occurs more commonly in post-natal calves, and early generalisation is more frequent than in adult cattle.

Tuberculous lesions in muscle, which occur subsequent to haematogenous or lymphatic spread, are extremely rare, possibly because muscle does not provide a favourable habitat for localisation and growth of tubercle bacilli (Tammemagi and O'Sullivan, 1955).

POSSUMS

Early field studies of populations of tuberculous possums (Cook, 1975; Cook and Coleman, 1975; Lake, 1975; Hickling *et al.*, 1991) provided information on the nature and distribution of macroscopic lesions (Table 1.3), and formed the basis for future cross-sectional studies. Although there were differences in the nomenclature of lymph nodes used by these and other (Julian, 1981; Coleman, 1988) authors, comparisons in the distribution of macroscopic lesions could still be made. The following is a list of terms used, and the terminology used in current studies and in Table 1.3:

Former name(s) used for lymph nodes	Current name of lymph nodes (Kampmeier, 1969)
Post-scapular	Deep axillary
External iliac, lumbar	Inguinal
Atlantal	Parotid
Retropharyngeal, peripharyngeal	Deep cervical
Pre-scapular, caudal cervical	Superficial cervical
Bronchial	Anterior mediastinal

Julian (1981) summarised the work of the authors below, describing macroscopic lesions in lymph nodes as being characterised by enlargement and softening, and frequently containing lime-green caseous pus. Lesions in visceral organs such as the lung and liver, consisted of soft cream-coloured foci or nodules ranging from 1 to 40 mm in diameter. Lung consolidation was also observed. Microscopically, the lesions have been described as pyogranulomatous in nature, and expansive with areas of caseation. Giant cells were not common and AFOs were often

present in high numbers. Mineralisation and fibrosis were not features of tuberculosis in possums (Lake, 1975).

Table 1.3 Distribution of macroscopic lesions in possums from field studies (percentage)

Lesion site*	n = 26[a]	n = 154[b]	n = 147[c]	n = 76[d]
Axillary ln	8	53	31	38
Inguinal ln	15	18	12	22
Parotid ln	-	-	1	-
Deep cervical ln	-	14	2	-
Lung	80	65	55	53
Ant. mediastinal ln	15	20	5	-
Mesenteric ln	27	20	18	21
Gastric ln	15	1	1	-
Hepatic ln	12	9	9	-
Liver	46	13	19	21
Kidneys	8	4	12	7
Spleen	8	6	10	6
Adrenal glands	8	-	2	-
GIT	8	-	-	-
Mammary glands	-	1	-	-
Internal iliac ln	4	3	5	-

*ln = lymph node; Ant. = anterior; GIT = gastrointestinal tract
[a]Waikato, 1974 (Lake, 1975; Julian, 1981)
[b]Buller and Inangahua, 1970-1974 (Cook, 1975; Julian, 1981)
[c]Hohonu, 1973-1974 (Cook and Coleman, 1975; Julian, 1981; Coleman, 1988)
[d]Hauhungaroa, 1982-1983 (Anonymous, 1986; Hickling *et al.*, 1991)

DEER

Most reports of tuberculosis in feral deer relate to red deer. However, *M. bovis* has been isolated from tuberculous lesions in three feral sika deer (*Cervus nippon*) (de Lisle and Havill, 1985; Nugent and Proffitt, 1994) and two feral fallow deer (*Dama dama*) (de Lisle and Havill, 1985). Probable tuberculosis affecting the thoracic cavity occurred in a further seven feral sika deer (Nugent and Proffitt, 1994).

In feral red deer the characteristic macroscopic lesions of *M. bovis* infection are soft, flocculent abscessed lymph nodes containing creamy pus. Primarily, lymph nodes of the head and neck are affected, some of which may discharge their contents to the exterior through sinuses. Pleuritis, sometimes in conjunction with pleural "grapes" (translucent fleshy outgrowths), is not uncommon in tuberculous feral deer (Lugton *et al.*, 1998). However, because of the great

diversity of tuberculous lesions in deer, any calcified, caseo-calcified, caseous, caseo-purulent or purulent lesions should be considered suspicious of tuberculosis (Montgomery, 1995).

Although tuberculous lesions in deer are typically pyogranulomatous in nature, they show a diversity of histological patterns. Usually, they are characterised by caseation, mineralisation (often peripherally), giant cells, and moderate but variable numbers of AFOs. Lesions are also often purulent, containing numerous neutrophils. Fibrosis is not a feature of tuberculous lesions in feral deer (Montgomery, 1995).

Rhyan and Saari (1995) provide a good description of the differences in the histopathology of tuberculous lesions in fallow, sika and red deer, and elk (*Cervus elaphus nelsoni*). Lesions in elk/red deer usually had scattered peripheral mineralisation rather than centralised mineralisation, and contained more neutrophils and fewer giant cells than did lesions in cattle. Lesions in fallow deer had more giant cells than in lesions in elk, whereas giant cells were most numerous and neutrophils least numerous in lesions of sika deer. Giant cells in lesions of sika deer were characterised as larger and containing more nuclei than giant cells in tuberculosis in the other deer species.

FERRETS

Macroscopic tuberculous lesions in ferrets are usually unspectacular, consisting merely of enlargement of (mesenteric) lymph nodes, which upon sectioning, often contain subcapsular multiple cream-coloured foci ranging from "pin-head" through to larger coalescing necrotic lesions (Lugton *et al.*, 1997b). Occasionally, entire mesenteric lymph nodes may undergo liquefactive change.

Microscopically, the lesions in this species are comprised of discrete aggregates of macrophages, with large aggregates centred on necrosis and surrounded peripherally by a thin band of lymphocytes and a few plasma cells. Fibrosis, mineralisation and giant cells are not present, and neutrophils are infrequent. Acid fast organisms have been recorded in large numbers in tuberculous lesions in ferrets (de Lisle *et al.*, 1993). However, Lugton *et al.* (1997b) recorded the occurrence of AFOs in lesser numbers in smaller lesions, increasing in number proportional to the increasing severity of granulomatous inflammation and necrosis. A characteristic feature of tuberculosis in ferrets is the occurrence of micro-granulomas in the hepatic parenchyma (Dunkin *et al.*, 1929; Symmers *et al.*, 1953; Anonymous, 1982). Lugton *et al.* (1997b) reported a 94% frequency of hepatic micro-granulomas in tuberculous ferrets, despite the lack of macroscopic lesions in the liver.

STOATS

Tuberculosis has been recorded in stoats (*Mustela erminea*) by Coleman (1975), Allen (1991) and Ragg *et al.* (1995a), but no details concerning the nature or distribution of lesions have been provided. Two tuberculous stoats were captured in 1992 and 1993 from a study site on the west coast of the South Island (Coleman *et al.*, 1994a). Both stoats had enlarged tuberculous mesenteric lymph nodes, suggesting infection via the oral route. In one node, the lesions were undergoing mineralisation. The other stoat had two small white spots in the spleen.

Macrophages predominate in tuberculous lesions in stoats, and they are arranged in discrete granulomatous foci, with neutrophils often admixed. In the mineralised mesenteric lymph nodes of one stoat some giant cells and fibrosis were also observed (Cooke *et al.*, 1999a).

WEASELS

The evidence for *M. bovis* infection in weasels (*Mustela nivalis*) is anecdotal only (Allen, 1991).

PIGS

Mycobacterium avium is the most common isolate (91%) from tuberculous domesticated pigs, whereas *M. bovis* has been isolated from almost all feral pigs (99%) with tuberculosis (de Lisle, 1994). Macroscopic lesions are largely confined to lymph nodes, particularly submandibular lymph nodes, and consist of nodular foci of caseous necrosis with central calcification. Fibrous encapsulation is prominent (Francis, 1958; Montgomery, 1995). Microscopic lesions in feral pigs infected with *M. bovis* are characterised by fibrous encapsulation, often extensive caseation with or without central mineralisation, few AFOs, and no giant cells (Montgomery, 1995).

CATS

Although *M. bovis* was isolated from 76 cats in a recent study (de Lisle, 1993), all but three were domesticated. A feral cat was described as having a typical tuberculous lesion in a mesenteric lymph node, although *M. bovis* culture was unsuccessful (Walker *et al.*, 1993). Ragg *et al.* (1995a) reported two tuberculous cats from the Otago and Southland regions of New Zealand, but no detail was provided concerning the location and microscopic nature of the lesions.

The most common type of lesion seen is in the skin, and is often mistaken for a cat bite abscess, but does not have the offensive smell typical of a cat bite abscess, and is intractable to conventional therapy (de Lisle *et al.*, 1990). A range of microscopic lesions from discrete granulomas with peripheral fibroplasia to large poorly defined coalescing lesions has been observed. Central caseous necrosis is common, sometimes undergoing mineralisation, and it is

in the necrotic areas that AFOs are most usually found in moderate to large numbers. The caseous foci are surrounded by macrophages and epithelioid cells, admixed with variable numbers of neutrophils. Giant cells are not a feature of tuberculous lesions in cats (pers. obs.). The lesions differ from those due to cat leprosy, which is characterised by macrophages containing massive numbers of AFOs, often arranged in parallel bundles, and numerous giant cells (Yager and Scott, 1993).

DOGS

In world literature reports, most cases of tuberculosis in dogs (*Canis familiaris*) are due to infection with *M. tuberculosis*, from close association with their owners. In New Zealand, very few cases of *M. bovis* infection of dogs have been reported, which may be a reflection of the dog's natural resistance to infection with this organism (de Lisle, 1993). Infection usually results from the association of (farm) dogs with tuberculous possums (de Lisle, 1993).

Macroscopically, white nodules range from soft through firm to hard, and as they are seldom yellow or caseous may be mistaken for sarcomas (Dodd, 1952). Gay *et al.* (2000) recently reported a case of pulmonary *M. bovis* infection in a German shepherd. The animal had a pleural effusion, pneumothorax, and an area of pulmonary consolidation. Histopathological examination of the surgically resected section of affected lung revealed numerous coalescing granulomas, containing central coagulative necrosis, surrounded by a wide zone of macrophages and occasional neutrophils, but no giant cells.

SHEEP

Macroscopic lesions may resemble those seen in cattle (Davidson *et al.*, 1981), or may be mistaken for caseous lymphadenitis (Cordes *et al.*, 1981). Tuberculous lesions of sheep have a very similar microscopic appearance to those of cattle (Davidson *et al.*, 1981).

GOATS

The macroscopic lesions seen in feral goats have been reported as being largely confined to the lung and lymph nodes of the head and thoracic cavity. Lesions were described as being often encapsulated and containing yellow caseous material (Sanson, 1988). Giant cells are present in large numbers in tuberculous lesions of goats. They are similar to those reported in hedgehogs, horses and sika deer. However, only in goats is the pattern of giant cells encircling central caseation observed to the same degree as occurs in cattle (Thoen, 1994). Sanson (1998) reported low to moderate numbers of AFOs in the lesions. Mineralisation of lesions was not a reported feature, and there was no mention of fibrosis.

HEDGEHOGS

The early record of lung lesions in a tuberculous hedgehog (Brockie, 1990) was followed more recently by the culture of *M. bovis* from four hedgehogs (Lugton *et al.*, 1995). All four hedgehogs infected with *M. bovis* had firm grey homogeneous 2-6 mm nodules in their lungs. One animal also had enlarged bronchial lymph nodes. Another animal with generalised tuberculosis had an enlarged, caseous, mineralising left retropharyngeal lymph node, an abscess in the right kidney and the right lobe of the prostate, an enlarged right mandibular lymph node, and a small number of pin point grey foci over the surface of the liver.

The pulmonary lesions in hedgehogs are composed of focal aggregates of macrophages interspersed with numerous giant cells and lesser numbers of lymphocytes and plasma cells, and occasional neutrophils. Necrosis, mineralisation and fibrosis are minimal or absent in these lesions, but are common in the retropharyngeal lymph node and prostate (Lugton *et al.*, 1995). Neutrophils are numerous in the renal lesions. Acid fast organisms are variable in number in pulmonary lesions but are moderate to high in number in other lesions in the generalised disease.

HORSES

Tuberculosis in horses is more commonly due to infection with *M. avium*, rather than *M. bovis*. Lesions are usually found in the abdominal cavity, particularly the mesenteric lymph nodes and spleen, and macroscopically the lesions are often described as sarcomatous in nature, as they are multilobular, firm and fibrous in texture (Francis, 1958). Microscopically, proliferative, fibrous, non-caseating lesions with numerous scattered giant cells, are characteristic of the disease. However, diagnosis is difficult if AFOs are not encountered (Innes, 1949).

HARES

After the first report of naturally occurring *M. bovis* infection in a free-living hare (Cooke *et al.*, 1993), a second tuberculous hare was trapped on the same study site, approximately 280 metres from the first animal (Coleman *et al.*, 1994a). Both animals had similar lesions, notably a chronic granulomatous pleurisy and small white spots throughout the pulmonary parenchyma. In the first hare, the mesenteric lymph nodes were enlarged and caseous, and a single 1-2 mm white nodule was present in the kidney and liver.

Discrete, focal granulomatous lesions, many of them with a caseous centre, and occasionally also with central mineralisation, were observed histologically on the pleural surface and in the pulmonary parenchyma (Cooke *et al.*, 1993). Large macrophages, epithelioid cells and a few

giant cells characterised these lesions, which were enclosed by a thin loose band of fibroblasts. Acid fast organisms were low in number, most numerous in necrotic or caseous areas.

RABBITS

Rabbits are highly susceptible to *M. bovis* infection under experimental conditions, and reasons for the apparent rarity of tuberculosis in wild rabbits remain a matter for speculation. Although naturally occurring tuberculosis has only ever been reported in one free-living animal (Gill and Jackson, 1993), it has been recorded in one of five rabbits in Ireland (Sleeman, pers. comm.). In this latter case, the rabbits came from area adjacent to a badgers' sett containing tuberculous animals. The rabbit population also had myxomatosis, and it is possible there may have been a link between an immunocompromised state due to myxomatosis and the occurrence of tuberculosis.

In the case reported in New Zealand (Gill and Jackson, 1993), macroscopic lesions consisted of enlargement and central caseation of the left prescapular lymph node, pulmonary consolidation, two pin-head pale foci in the liver, and many small foci throughout the renal cortices. The histological lesions were similar to those in hares, with central necrosis surrounded by epithelioid and mononuclear inflammatory cells. Giant cells were reported only in pulmonary lesions. Variable numbers of AFOs were present in all affected organs. Moderate numbers were seen in the liver and kidney.

1.5.2 The pathology of tuberculosis in mammals in overseas countries

A diverse range of mammalian species has been reported with *M. bovis* infection and these are listed in Table 1.2. Some of the infected animals were held in zoos, but all were naturally infected with *M. bovis*, and it is difficult to generalise about the nature of the lesions encountered. Because of difficulties in animal handling, it is likely that the majority of these animals would not have been detected until in the terminal stages of the disease, or following death from overwhelming disease.

Many of the bovine, camel, antelope and cervine species show similar histopathological characteristics to those described in *M. bovis* infection of cattle. In most other species, however, giant cells are rare or do not occur in tuberculous lesions, apart from those in the ass (Thoen, 1994), and AFOs are recorded as occurring in high numbers in almost all of these species.

Table 1.4 Miscellaneous mammals confirmed as culture-positive for *Mycobacterium bovis*

Species	Reference(s)
ARTIODACTYLA:	
Bovidae:	
Antelopes:	
Blackbuck (*Antilope cervicapra*)	cited by Zuckerman, 1980
Bushbuck (*Tragelaphus scriptus*)	cited by Zuckerman, 1980
Cape duiker (*Silvicapra grimmia*)	Griffith, 1939; Paine and Martinaglia, 1928
Eland (*Taurotragus oryx*)	Thoen *et al.*, 1977; Krauss *et al.*, 1990
Gnu (*Connochaetes taurinus*)	Griffith, 1939
Impala (*Aepyceros melampus*)	De Vos *et al.*, 1977
Kudu (*Tragelaphus strepsiceros*)	Griffith, 1939; Thoen *et al.*, 1977, 1995
Lechwe/marsh antelope (*Kobus leche*)	Clancey, 1977; Thoen *et al.*, 1995
Nilgai (*Boselaphus tragocamelus*)	Thoen *et al.*, 1995
Nyala (*Tragelaphus angasi*)	Hofmeyr, 1956
Sable-horned antelope (*Hippotragus niger*)	Thoen *et al.*, 1995
Sitatunga (*Tragelaphus spekei*)	Thoen *et al.*, 1977
Bovine:	
Balinese (banteng) cattle (*Bos sondaicus*)	Tweddle and Livingstone, 1994
Camelidae:	
Llama (*Lama glama*)	Thoen *et al.*, 1995
Cervidae:	
Black-tailed deer (*Odocoileus hemionus columbianus*)	Hunter, 1996
Hog deer (*Axis porcinus*)	Basak *et al.*, 1975
Moose (*Alces alces*)	Hunter, 1996
Sambar deer (*Cervus unicolor*)	Liston and Soparkar, 1924
Giraffidae:	
Giraffe (*Giraffa camelopardalis*)	Nieberle, 1938
Suidae:	
Pot-bellied pig (*Potamochoerus larvatus*)	Essey and Vantiem, 1995
CARNIVORA:	
Canidae:	
Coyote (*Canis latrans*)	Hunter, 1996; Bruning-Fann *et al.*, 1998*
Jackal (*Canis cancrivorus*)	Griffith, 1928a
Felidae:	
Lynx (*Felis lynx*)	Schliesser, 1976
Tiger (*Panthera tigris*)	Griffith, 1928a; Schliesser, 1976
Viverridae:	
Civet (*Viverra civetta*)	Griffith, 1928a
INSECTIVORA:	
Talpidae:	
Mole (*Talpa europaea*)	cited by Lepper and Corner, 1983*
MARSUPIALIA:	
Macropodidae:	
Kangaroo (*Macropus* spp.)	Huitema, 1972
PERISSODACTYLA:	
Equidae:	
Ass (*Equus hemionus*)	Thoen, 1994
Tapiridae:	
Tapir (*Tapirus terrestris*)	Griffith, 1939; Thoen *et al.*, 1977
PROBOSCIDAE:	
Elephantidae:	
Indian elephant (*Elephas maximus*)	Thoen, 1994
RODENTIA:	
Hydrochoeridae:	
Capybara (*Hydrochoerus* spp.)	Huitema, 1972
Sciuridae:	
Ground squirrel (*Citellus beecheyi*)	Griffith, 1939

**M. bovis* was cultured, but no lesions were evident

1.5.3 Miscellaneous records of tuberculosis in mammals

Table 1.4 contains a list of mammals in which *M. bovis* has been recorded, but for which no details have been reported with regard to lesion distribution, possible routes of infection or histopathological features of the disease.

1.6 EXPERIMENTAL INFECTIONS WITH *MYCOBACTERIUM BOVIS*

1.6.1 Early experimental studies in possums

Prior to the discovery of tuberculosis in possums in New Zealand, Bolliger and Bolliger (1948) conducted experimental infections of possums in Australia, in order to study the susceptibility of marsupials to some of the pathogens of eutharian mammals. A total of 10 possums were infected with either *M. tuberculosis* or *M. bovis*, via the intra-peritoneal (I/P), intra-muscular (I/M), or oral routes. Death ensued 2-5 weeks post-inoculation (p.i.), with dissemination of lesions in the lung and abdominal cavity. Oral inoculation of two possums, one with lung and the other with an I/P exudate, from infected possums resulted in death from generalised tuberculosis 2 and 7 months later (respectively). It was claimed that aerosol transmission occurred in a non-infected possum which shared a cage with an experimentally infected possum, but as physical contact between the two possums was possible, a conflicting variable was introduced. However, the experiments did show that possums might become naturally infected from other possums.

In New Zealand, O'Hara *et al.* (1976) recorded the distribution of lesions produced 25-100 days p.i. following infection of possums via S/C and intra-nasal (I/N) routes with possum and cattle isolates of *M. bovis* (Table 1.5). A different pattern of lesion distribution between the two routes of infection was observed.

Most possums placed in direct contact (3 of 4), close contact (2 of 3), or in a situation where aerosol infection might occur (2 of 3), developed pulmonary and/or generalised tuberculosis and most subsequently died. One of the possums in direct contact was a joey suckling its mother. Lesions in GIT sites suggested infection occurred via the oral route.

Table 1.5 Distribution of macroscopic lesions in possums after subcutaneous and intranasal inoculation with *Mycobacterium bovis

	Percentage of lesions in possums									
Inoc. Route	L & R ax ln	L & R ing ln	Head Ln	L & R lung	Mesen ln	Hep ln	Liver	L & R kidney	Spleen	L & R adrenal
S/C	57	86	14	100	71	100	100	57	100	14
I/N	0	0	100	88	100	75	75	13	75	0

*Compiled from work of O'Hara *et al.* (1976)
Inoc. = inoculation; L = left; R = right; ax = axillary; ln = lymph node; ing = inguinal; mesen = mesenteric; hep = hepatic; S/C = subcutaneous; I/N = intra-nasal

Results of experiments conducted in Australia by Corner and Presidente (1980, 1981) using an Australian cattle strain (B_1 and/or B_2) of *M. bovis*, and a strain derived from tuberculous possums from New Zealand (P), are summarised in Table 1.6.

Table 1.6 Presence of macroscopic lesions in possums following inoculation with *Mycobacterium bovis* via the intra-muscular route. (Compiled from work of Corner and Presidente (1980, 1981))[a]

No. of weeks p.i.	Deep ax ln	Inguinal ln	Lung	Mesen ln	Liver	Hepatic ln	Kidney	Spleen	GIT
2	-	-	-	-	-	-	-	-	-
4	P	B_1B_2P	P	B_2P	B_2P	P	P	B_2P	P
6	-	B_1	B_1	B_1	B_1	-	B_1	B_1	B_1
8-10	B_2P	B_1B_2P	B_1B_2P	B_1B_2P	B_1B_2P	B_2P	B_1B_2	B_1B_2P	B_1B_2

[a]Dose rate and strain of *Mycobacterium bovis* used:
P = 10^4 bacilli from a possum from New Zealand
B_1 = 10^7 bacilli from cattle from Australia
B_2 = 10^3 bacilli from cattle from Australia
p.i. = post-inoculation; ax = axillary; ln = lymph node; mesen = mesenteric; GIT = gastrointestinal tract

Apart from inoculation site abscesses, no macroscopic lesions were observed at 2 weeks p.i. A lower dose of the strain derived from the tuberculous possum in New Zealand was used than one of the doses (B_1) of the strain derived from cattle from Australia. However, more lesions were produced, particularly at 4 weeks p.i., using the strain derived from the tuberculous possum, suggesting this strain was more virulent than the other.

1.6.2 Recent experimental studies in possums

Over the last 10 years, there has been renewed interest in reproducing an experimental model of tuberculosis in possums in New Zealand which mimics the natural disease. In order to establish

an appropriate dose-rate, Buddle *et al.* (1994) injected five possums each with high (2×10^5 colony forming units (cfu)), medium (2×10^3 cfu), and low (20 cfu) doses of *M. bovis* via the intra-tracheal (I/T) route. The trachea was approached through the skin of the ventral mid-neck, and this resulted in needle-track abscesses in 60%, 80% and 100% of the possums in the low, medium and high dose groups (respectively). Possums in the high and medium dose groups were humanely killed before completion of the experiment at 60 days p.i. (range 33-57 and 43-57 days, high dose and medium dose groups respectively). A wide range of tissues was examined, even though macroscopic lesions were not present at all the sites selected for histopathology. This work demonstrated lesions at more distant sites than was indicated macroscopically. All possums had lesions in deep cervical lymph nodes (Table 1.7). Lesions were also common in axillary, superficial cervical and hepatic lymph nodes, and less so in mesenteric, mandibular, renal, inguinal, parotid, internal iliac and gastric lymph nodes, with possums in the high and medium dose groups having a higher proportion of lymph nodes affected. Lobar consolidation of the lung and miliary tuberculosis were more common in possums in the high dose group than in the low dose group. *Mycobacterium bovis* was cultured from the lung of one of five in-contact possums, none of which had macroscopic evidence of lesions when they were killed at 9 weeks p.i. The authors suggested that had these possums been allowed prolonged contact with the inoculated possums, aerosol transmission of infection may have occurred.

Table 1.7 Percentage of possums with macroscopic lesions following intra-tracheal inoculation with *Mycobacterium bovis**

Group[a]	Deep cervical ln[b]	Lung	Liver	Kidney	Spleen
Low	100	100	20	20	20
Medium	100	100	20	40	60
High	100	100	20	80	80

*Compiled from work by Buddle *et al.* (1994)
[a]Low = 20 colony forming units (cfu), medium = 2×10^3 cfu, high = 2×10^5 cfu
[b]ln = lymph nodes

Histopathologically, the experimental lesions showed an extensive pyogranulomatous inflammatory reaction, central necrosis, few giant cells, rare fibrosis, and no mineralisation. Acid fast organisms were numerous in necrotic areas and in the cytoplasm of macrophages and giant cells around the periphery. Organism numbers increased with increasing size of the lesions.

Following on from the experiments of Buddle *et al.* (1994), Pfeffer *et al.* (1994) selected a low dose (125 cfu) of *M. bovis* when they inoculated six possums via the endo-bronchial (E/B)

route. The inoculum was dispensed via a cannula inserted *per os* into the larynx and deep into the tracheal lumen, circumventing problems encountered previously with needle-track infections. A wide range of sites was examined after the animals were killed at 55-57 days p.i. (Table 1.8).

Table 1.8 Distribution of lesions produced in six possums inoculated via the endo-bronchial route with *Mycobacterium bovis**

	Number of possums which showed lesions[a]														
	Axillary	Inguinal	Tonsil	Mandib	Parotid	Deep cervical	Super cervical	Lung	Anterior medias	Mesen	Gastric	Hepatic	Liver	Kidney	Spleen
Macro	0	0/5	0	0	0	0	0	6	6	0	0/3	3	3	2	4
Histo	5	4/5	1/5	5	1/2	6	2	6	6	6	3/3	6	6	6	5/5

*Compiled from work by Pfeffer *et al.* (1994)
Mandib = mandibular; Super = superficial; medias = mediastinal; Mesen = mesenteric
Macro = Macroscopic; Histo = Histopathology
[a]Denominators are used where tissues from fewer than six possums were examined

The histopathological picture and numbers of AFOs were similar to that described by Buddle *et al.* (1994). The authors suggested several routes of dissemination of infection throughout the body, such as clearance of pulmonary mucus may lead to infection of the GIT, and lesions produced in peripheral nodes indicated haematogenous spread. It was concluded that the E/B route of infection was useful as a model of the natural disease, but that the dose rate used for this purpose may have been too high.

In these later experiments, histopathological examination of a wide range of tissues allowed detection of lesions not evident macroscopically. However, because the animals were killed when lesions were advanced, the experiments gave little indication of the pathogenesis of the disease. Nevertheless, because the E/B route of infection produced lesions similar to those that occur naturally, the experiments established a model suitable to study the pathogenesis of tuberculosis in possums.

1.6.3 Recent experimental studies in maintenance hosts

In their review of recent experimental work in cattle, Griffin and Dolan (1995) concluded that the studies indicated that infected cattle can excrete *M. bovis* at various stages following infection, but it was the number of organisms excreted and/or the size of infective particles which may be the crucial factor(s) in setting up infection in in-contact cattle, either in that herd or in an adjoining herd. More recent experimental infection of cattle has been directed at

studying early lesion formation following intranasal (Cassidy *et al.*, 1998) or intra-tonsillar (Palmer *et al.*, 1999b) inoculations with *M. bovis*. Both the nasal cavity and tonsil are probable important routes of infection in the natural disease in cattle, and information derived from these experiments helped an understanding of the pathogenesis of tuberculosis in this species, and offers a feasible explanation for the occurrence of reactors with no visible lesions during routine meatworks inspection procedures.

Similar experimental work involving intra-tonsillar inoculation of red deer (Mackintosh *et al.*, 1995) and white-tailed deer (Palmer *et al.*, 1999a) has produced a good model of naturally occurring tuberculosis in deer. The model has also been used to study the immunology and genetics of resistance to tuberculosis, with the view to selecting tuberculosis resistant stags for breeding to enhance herd immunity (Mackintosh *et al.*, 2000).

In England, the Krebs report (1997) concluded that although strong evidence exists to demonstrate an association between infection in badgers and cattle, the transmission of the disease is yet to be formally proven. Investigation of the consequences of badger removal has demonstrated perturbation effects, which has epidemiological consequences for tuberculosis transmission within socially disturbed badger populations (Macdonald *et al.*, 2000). These two recent developments, coupled with legislation which protects badgers, are essentially why experimental infections of badgers in England have foundered, and emphasis has shifted to mathematical models (Gallagher *et al.*, 2000), and implementation of husbandry methods aimed at reducing the risk of infection. In Ireland, however, experiments are being conducted, which are aimed at establishing a suitable route of vaccination of badgers, which would ultimately result in a tuberculosis free population of badgers, and thus limit spread to livestock and other species (Sleeman, 2000; Southey *et al.*, 2000).

1.7 SUMMARY AND CONCLUSIONS

Tuberculosis caused by *Mycobacterium bovis* has been reported in an exceptionally wide range of mammals, and in New Zealand is maintained in its primary host, cattle, as well as deer and possums. The possum is the wildlife reservoir host for the disease in this country, a rôle analogous to that played by the badger in Great Britain and Ireland.

In the majority of mammalian species the respiratory tract is the most important route of *M. bovis* infection. This route involves the deposition of droplet nuclei in the lower airways, and consequent establishment of pulmonary tuberculosis, the lesions of which in some

ruminants are frequently found in peripheral dorsocaudal areas of lung. Although the holding of animals in close confinement would facilitate aerosol spread of infection, the build-up of infectious secretions and excretions in the environment would also increase the potential for infection via the oral route. However, the actions of heat and sunlight, such as prevails during summer, would minimise the risk of infection, due to the reduced survival of the organism in the environment, particularly on fomites.

Due to the limited use of laboratory diagnostic techniques, and limited and non-standardised methods used to capture data, field studies of naturally occurring tuberculosis underestimated the level of infection within populations of possums (Hickling *et al.*, 1991). Descriptions of the pathology of the disease were confined to those animals with generalised and/or advanced disease, and did not describe small and/or early lesions, nor give any indication of the spread or development of the disease. They also failed to resolve the issue of a high frequency of lesions in superficial lymph nodes, although some workers did suggest that the percutaneous route may be responsible for lesions developing at these sites. Information available concerning percutaneous infection of other animal species suggests that few organisms are required for the establishment of disease via this route.

Most experimental infections of possums have been of a fixed duration, which meant they did not follow the full course of the disease, and those which were sequential involved too few animals for statistically significant conclusions to be made. Later experiments were useful in establishing an appropriate dose rate to administer when investigating tuberculosis. Despite experimental infections via I/M and S/C routes, neither of which is likely to occur in the natural disease, no experiments have been conducted using the I/D route, which may be the key to explaining the presence of lesions in superficial lymph nodes.

CHAPTER 2. THE MORPHOLOGY OF THE LUNG OF THE BRUSHTAIL POSSUM

2.1 INTRODUCTION

In most mammalian species, infection with *Mycobacterium bovis* occurs via the respiratory route, resulting in pulmonary tuberculosis as the most common manifestation of the disease. Airborne infection occurs by spread of droplet nuclei and dust (Langmuir, 1961). Primary pulmonary tuberculosis begins when a droplet nucleus (0.5 to 3 μm in diameter) containing one or two viable tubercle bacilli is inhaled and deposited on the alveolar surface where the organisms begin to multiply (Bates, 1980). Alveolar macrophages are the main defence mechanism in the distal airways, but phagocytosis of tubercle bacilli is relatively ineffective, as the ingested bacteria may remain viable and continue to replicate. The structure of the lower respiratory tract of different species will affect particle deposition through turbulence. Furthermore, the efficiency and distribution of defence mechanisms such as the mucociliary apparatus and lymphoid aggregates will play an important rôle in determining the fate of inhaled organisms.

The feral brushtail possum (*Trichosurus vulpecula*) in New Zealand is known to be highly susceptible to *M. bovis*. It has been observed that the lung is a commonly affected site for naturally occurring tuberculosis in wild possums (Julian, 1981; Coleman, 1988). Although the tubercle bacillus appears to have a propensity for this organ, the pathogenesis of tuberculosis in the lung of the possum is poorly understood, and the possibility that respiratory defence mechanisms in the possum are structurally inadequate requires investigation.

There are few light and electron microscopic descriptions of the marsupial lung. Those which have been published have placed emphasis on the postnatal development of alveoli. These include studies of the American opossum, *Didelphis virginiana* (Sorokin, 1962; Krause and Leeson, 1973, 1975; Krause *et al.*, 1976), Australian eastern native cat, *Dasyurus viverrinus* (Hill and Hill, 1955), Australian northern native cat, *Dasyurus hallucatus* (Gemmell and Nelson, 1988), the red-necked wallaby, *Macropus rufogriseus* (Walker and Gemmell, 1983), the tammar wallaby, *Macropus eugenii* (Runciman *et al.*, 1996, 1999), the marsupial bandicoot, *Isoodon macrourus* (Gemmell and Little, 1982; Gemmell, 1986), and the brushtail possum (Gemmell and Nelson, 1988; Buaboocha and Gemmell, 1997). Only Krause and Leeson (1973,

1975) provide some information concerning the development of bronchi, and Tucker (1974) makes brief mention of the trachea and bronchi of the brushtail possum and koala (*Phascolarctos cinereus*). However, none of the documented studies of marsupial lungs to date covers the anatomical or morphological features of the entire lung.

This chapter describes a light and electron microscopic study of the morphology of the lung of healthy immature and adult brushtail possums, with particular emphasis on the mucosal epithelium of the airway conducting system. The study attempted to evaluate the relative importance of mucosal defence mechanisms in the lower respiratory tract of possums and relate this to eutharian mammals with a known tuberculosis susceptibility.

Time constraints necessitated a detailed study of the respiratory tree of the lung of the possum by means of dissection of the airways, rather than making a cast of the lung. Considerable knowledge and expertise are required when making casts of the conducting portion of the airways. The technique is complicated by the pressure of the lung, which is more difficult to assess in small lungs, such as the possum's. Additionally, the viscosity of the latex is manipulated according to the extent of definition required (Nutman, pers. comm.). As the light and electron microscopic studies would provide information on the finer structure of the airways, dissection of the major airways of the lung was adopted in favour of producing a cast.

2.2 MATERIALS AND METHODS

2.2.1 Animals

The study covered a total of 73 feral possums, ranging in age from pouch young (neonatal to 4 months), through back-riders (≥4 months) and immature possums (6-12 months), to sexually mature possums up to 5 years of age. They were captured in rural areas in the Manawatu region of New Zealand, which is free from endemic tuberculosis. The age of pouch young and back-riders was based on head length and body weight, using the guidelines of Lyne and Verhagen (1957), and Kingsmill (1962). Immaturity was determined by the time of year of capture, and the size of the gonads in males or absence of a pouch in females. Mature possums were determined by the size of the gonads in males or the presence of a pouch in females, and the pattern of tooth-wear on the upper left first molar, using the guidelines of Winter (1980).

Possums aged 4 months or older were anaesthetised with an intra-muscular injection of Ketamine HCl (Parnell Laboratories, New Zealand) at 25 mg/kg (Pfeffer *et al.*, 1994), followed by euthanasia using intravenous pentobarbitone. Euthanasia in younger possums was effected by intra-cardiac pentobarbitone injection. Necropsy was conducted immediately after death.

Two age groups of possums were studied. The lungs from one group of possums, comprising 27 pouch young and back-riders, ranging in age from neonatal to 5.5 months, were fixed in 10% neutral buffered formalin, for a light microscopic study of the development of the lung. Of the other group of 46 possums, the lungs from 40 animals were fixed in 10% neutral buffered formalin, for macroscopic anatomical and light microscopic studies. The lungs from the remaining six possums were used for electron microscopic studies.

2.2.2 Macroscopic anatomy

Following fixation in 10% neutral buffered formalin, the size and volume of each lung lobe from 10 possums were measured and recorded. After cutting away external cartilaginous structures such as the trachea and primary bronchi, the entire lung was submerged in a measuring cylinder filled with 10% neutral buffered formalin, and the total volume of fluid displaced recorded. Each lobe was then dissected free and individually submerged, and the subsequent displacement of fluid by each lobe was recorded.

The fixed lungs from 10 possums were carefully dissected along the airways, and a diagram drawn of the arrangement of both the major and minor intra-pulmonary airways.

2.2.3 Histology

Twelve immature and eight adult possums were used in this study. Longitudinal sections of lung were taken from each lobe at its base (hilus) nearest the lobar bronchus, and horizontal sections were taken midway across the lobe. Sections of lung were also taken from midway in the six lung lobes of the group of 27 pouch young and back-riders. Each section was embedded in paraffin and routinely processed and cut to produce 4 μm sections, which were stained by haematoxylin and eosin (H&E). Selected sections were also stained with alcian blue (AB),

pH 1[1], for sulphated acid mucins, and periodic acid Schiff-alcian blue (PAS/AB), pH 2.5[1], for acid mucopolysaccharides.

The sections were examined histologically for the presence of mucosal associated lymphoid tissue (MALT), the occurrence and site of any other lymphoid tissue, and the location of cartilage and submucosal glands. However, of the group of 27 pouch young and back-riders, only information concerning the development of lymphoid tissue, including MALT, is detailed here. From the other group of possums, the types of cells present in groups of 200-600 epithelial cells of the mucosae of the airways in each area of lung were identified and the proportion of goblet cells in the respiratory epithelium of bronchi and bronchioles was recorded.

2.2.4 Samples for electron microscopy

Samples of fresh lung from six mature possums were fixed in 3% gluteraldehyde. These were post-fixed in 1% osmium tetroxide in phosphate buffered saline (pH 7.4) for 1 hour and embedded in epoxy resin (Procure 812, Probing and Structure, Thuringowa, Queensland, Australia). Thick sections, stained with toluidine blue, were examined by light microscopy, to evaluate histological features of normal structure, and determine areas of interest for electron microscopy. Thin sections were cut and mounted on copper grids before staining with uranyl acetate and lead citrate, and the grids examined by transmission electron microscopy (TEM) (Philips 201c TEM).

2.3 RESULTS

2.3.1 Macroscopic findings

The lungs consisted of six lobes: the left and right cranial and caudal lobes, and the right middle and accessory lobes. Although none of these lobes was clearly divided, there was often a distinctive indentation of the cranial part of the left caudal lung lobe. Only one main branching lobar bronchus supplied each of the four smallest lung lobes (the left and right cranial, and the right middle and accessory), whereas the major branch to the left and right caudal lobes possessed four side branches towards the lateral sides of the lobes (Figure 2.1). A bronchial

[1]Culling CFA, Allison RT, Barr WT (eds). Cellular Pathology Technique. 4th Edtn. P 233. Butterworths, London, 1985.

branch, originating from the ventral side of the proximal end of the major branch of the bronchus supplying the left cranial lobe, provided a fifth side-branch in the cranial part of the left caudal lobe.

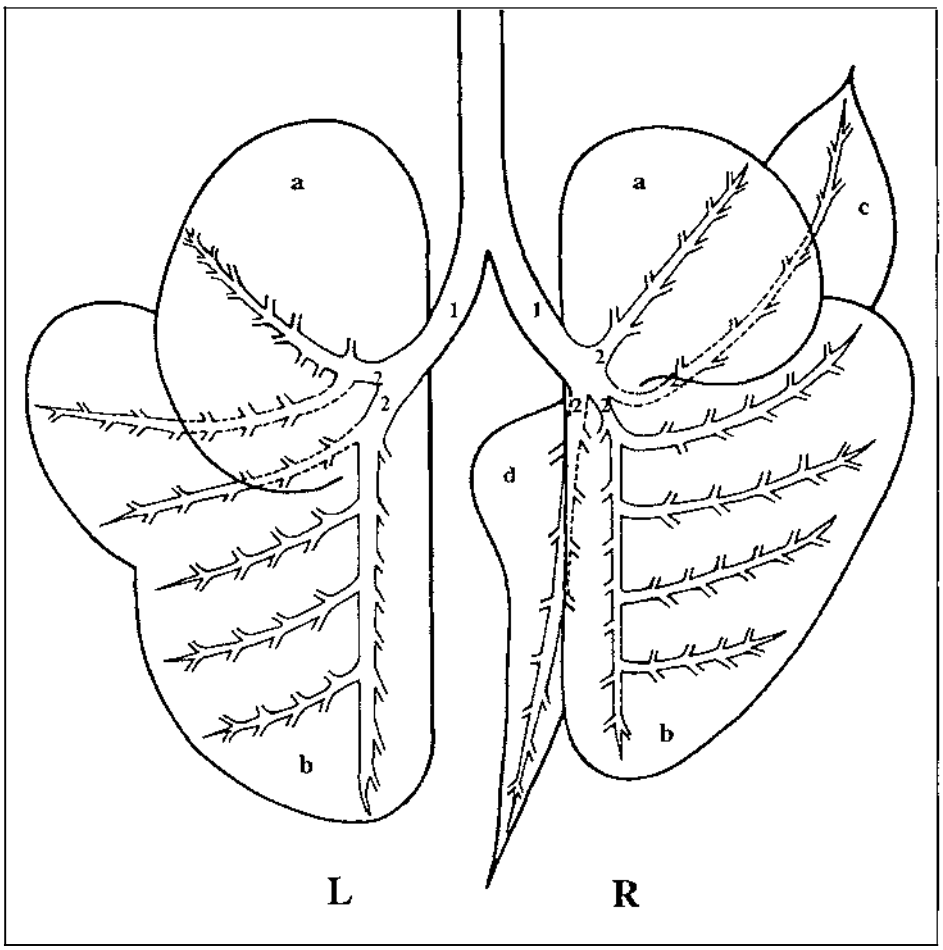

Figure 2.1 Proportionately scaled diagrammatic representation of the lung and lower airways of the possum lung. (L = left; R = right; 1 = primary bronchus; 2 = lobar bronchus; a = cranial; b = caudal; c = middle; d = accessory).

The mean length of the left cranial lobe was greater than that of the right cranial lobe, and the mean width of the left caudal lobe was greater than that of the right caudal lobe (Table 2.1). The left lobes of the lung varied more in shape than the other lobes, and this was reflected in variation of their dimensions. The volume of the right lung compared with the left was larger by a ratio of 4:3. The left and right caudal lung lobes comprised 33% and 32% of the total lung volume respectively, and each lobe was more than three times the volume of each of the other lobes. The right accessory lung lobe was the smallest lobe, comprising only 7.3% of the total lung volume. There was little variation in size or volume of the lung lobes either between the immature and mature possums, or between individuals.

Table 2.1 Range in size and volume of the lung lobes of possums

	Length (cm)		Width (cm)		Volume		
Lung lobe	Range	Mean	Range	Mean	Range (mL)	Mean (mL)	% of total
Left cranial	2.9-4.1	3.6	2.2-3.4	2.8	2.0-4.0	2.7	9.1
Left caudal	5.0-6.5	5.6	2.7-4.4	3.6	7.5-12.5	9.8	33
Right cranial	2.2-3.7	3.0	2.2-3.5	2.7	2.5-3.5	2.9	10
Right middle	3.1-4.6	4.1	1.7-2.7	2.2	2.0-3.5	2.6	8.6
Right caudal	4.0-6.4	5.6	2.9-3.5	3.1	7.5-12.5	9.6	32
Right accessory	3.7-4.5	4.2	1.6-2.6	2.2	1.75-2.75	2.2	7.3

2.3.2 Microscopy

BRONCHI

Primary bronchi had a thicker mucosal epithelium than secondary bronchi, and both were comprised of pseudostratified columnar cells. Lobar, or secondary, bronchi supplied each of the six lung lobes, and were located at the root of each lobe. Their luminal diameter ranged from 550-2000 μm, with a mean diameter of 1200 μm (Table 2.2). Cartilage and submucosal glands were present around the hilus of these bronchi as they entered the lobes of the lung (Figure 2.2). Cartilage continued for a distance of no more than 1 mm into the lung parenchyma, whereas submucosal glands continued for a distance of between 1-8 mm, after which neither was observed. Thus, the secondary bronchi did not divide into tertiary bronchi, so that the segments of each lobe were supplied by major bronchioles.

Table 2.2 Morphological features of the lower respiratory tract of the possum

	Range in luminal ∅ (Average) (μm)	Epithelial thickness	Ciliated cells	Clara cells	Goblet cells	Submucosal glands	Cartilage
Secondary bronchi	550-2000 (1200)	30 μm	33-50%	50-66%	<1% (1:200-600)	Yes	Yes
Major bronchioles	250-900 (530)	18.5 μm	≈ 30%	≈ 70%	Rare	No	No
Terminal bronchioles	150-550 (280)	11.5 μm	≈ 10-30%	≈ 70-90%	Absent	No	No
Respiratory bronchioles	50-150 (110)	7.0 μm	Absent	100%	Absent	No	No

∅ = diameter

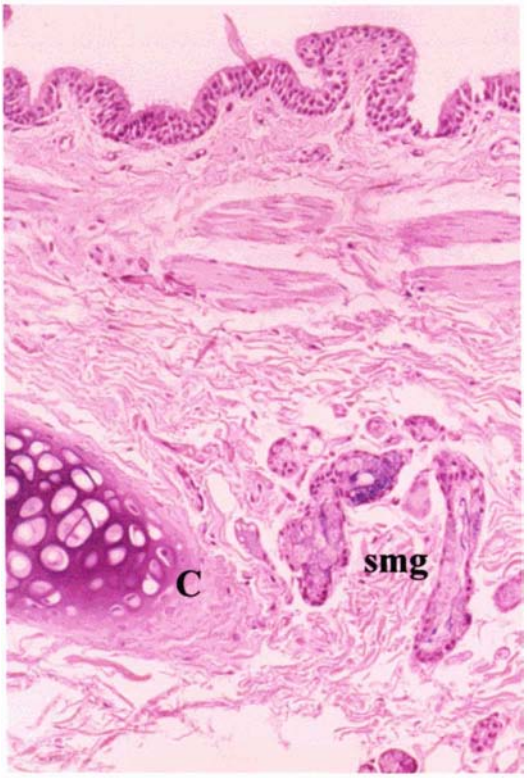

Figure 2.2 Submucosal glands (smg) and cartilage (C) are well developed at the hilus of each lobe in the mature lung. H&E. Magnification = 140x.

The two main cell types populating the columnar epithelium of the mucosa of the bronchi were ciliated cells, and non-ciliated cells, with basally located nuclei. These cells were interspersed with occasional intermediate cells, and basal cells were present at their base (Figures 2.3 and 2.4). The proportions of ciliated to non-ciliated cells varied from between 1:1 and 1:2. The non-ciliated cells frequently contained a distinct accumulation of material in the apices of their cytoplasm, which stained positively with PAS and negatively with AB at pH 2.5 (Figure 2.5), and the apex of the cells frequently projected into the lumen of the airway. By TEM, these non-ciliated secretory cells were identified as Clara cells, which were covered by microvilli, and had a cytoplasm rich in dense, ovoid secretory granules located in the apical cytoplasm (Figures 2.3 and 2.4). Goblet cells were rarely seen in the respiratory epithelium of the bronchi, occurring less than 1% (every 200-600 cells) in relation to other mucosal epithelial cells. The goblet cells stained positively by AB at both pH 1 and pH 2.5 (Figure 2.5), denoting the presence of sulphated glycoprotein.

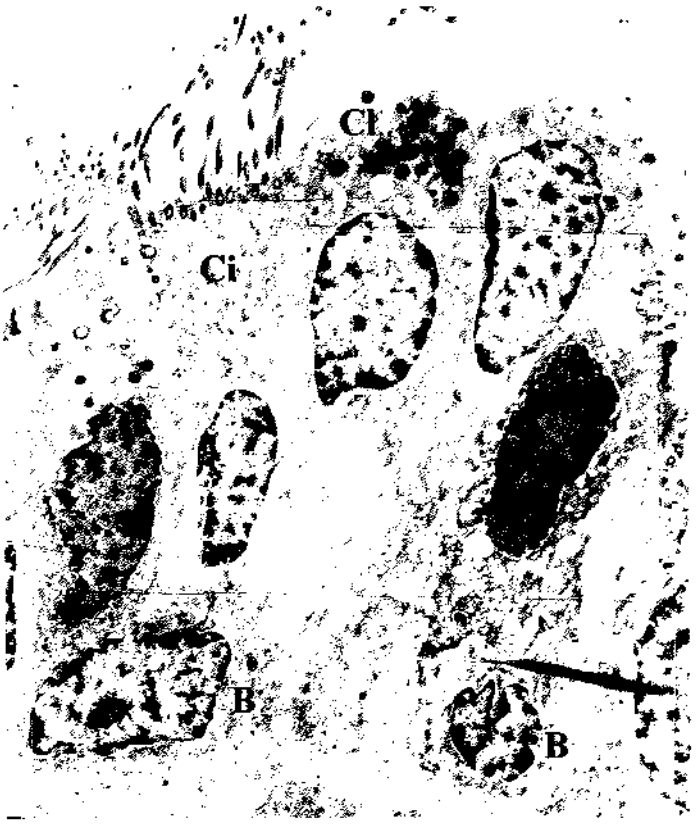

Figure 2.3 Primary bronchus, lined by ciliated (Ci) and non-ciliated Clara (Cl) cells, demarcated ventrally with basal cells (B). TEM. Uranyl acetate-lead citrate. Magnification = 7800x.

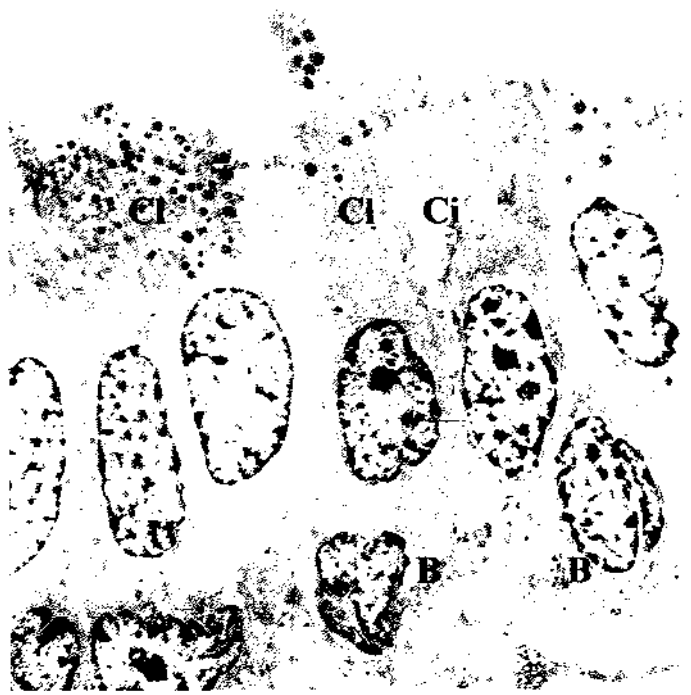

Figure 2.4 Secondary bronchus, consisting of ciliated (Ci) and non-ciliated secretory epithelial cells (Clara cells) (Cl), with basally situated basal cells (B). TEM. Uranyl acetate-lead citrate. Magnification = 5200x.

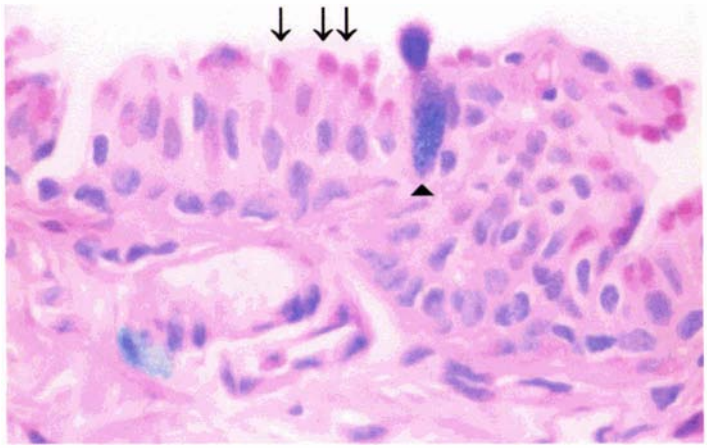

Figure 2.5 Goblet cells in the bronchial mucosal epithelium were easily identified (arrowhead). Special stains highlighted non-ciliated epithelial cells by the presence of red-staining material (neutral glycoprotein) in their apices (arrows). PAS/AB, pH 2.5. Magnification = 525x.

BRONCHIOLES

Major bronchioles branched into terminal bronchioles, then respiratory bronchioles, which opened into alveolar ducts (Figure 2.6). The two cell types lining the bronchiolar mucosae were ciliated and non-ciliated secretory (Clara) cells, the latter type increasing proportionately as the bronchioles branched and narrowed distally. Ciliated cells were not observed in respiratory bronchioles, and Clara cells were the only cell type present at this level. In the bronchiolar epithelium, the Clara cells were cuboidal, and their intra-cytoplasmic secretory granules were not always as distinctive as those observed in the bronchi (Figure 2.7). Goblet cells were extremely rare in the epithelium of major bronchioles, and were recorded 4-10 times less often than in the secondary bronchi. They were not observed in the mucosal epithelium below the level of major bronchioles. Brush cells were not seen, and basal cells were rarely observed.

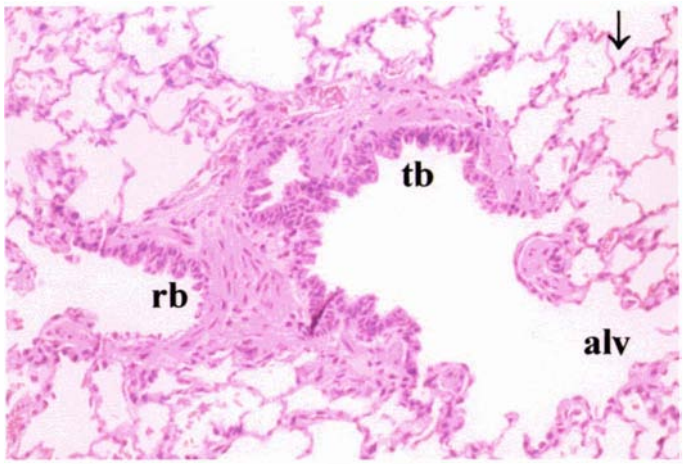

Figure 2.6 Distal portion of the conducting system, depicting a terminating bronchiole (tb), respiratory bronchiole (rb), an alveolar duct (alv), and a pore of Kohn (arrow). H&E. Magnification = 125x.

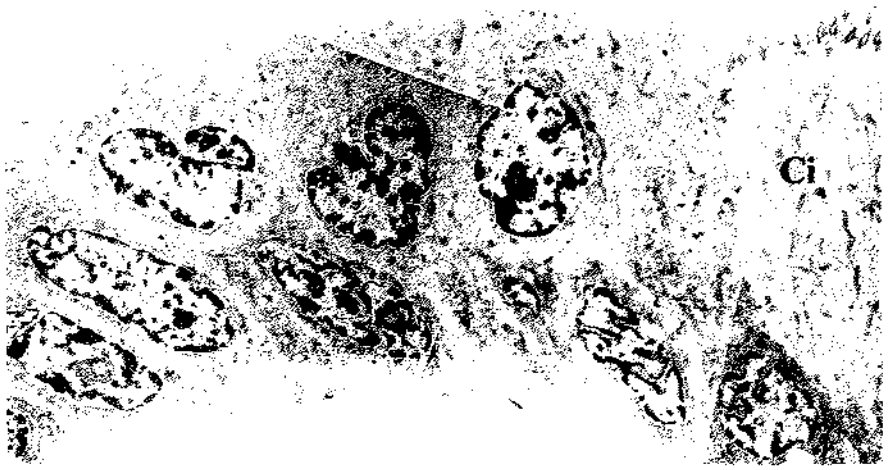

Figure 2.7 Terminal bronchiole. Non-ciliated epithelial cells, covered by microvilli, out-number ciliated cells (Ci) at least 4 to 1. TEM. Uranyl acetate-lead citrate. Magnification = 5200x.

ALVEOLI

The alveolar septa were typically thin-walled and well-vascularised (Figure 2.6). They were lined by both type I and type II alveolar cells, and there were between one and three alveolar macrophages per alveolar space. Type II cells contained large numbers of vacuolated, dark-staining, osmiophilic lamellated bodies (cytosomes) in their cytoplasm (Figure 2.8). Pores of Kohn were occasionally seen, but no brush cells were observed.

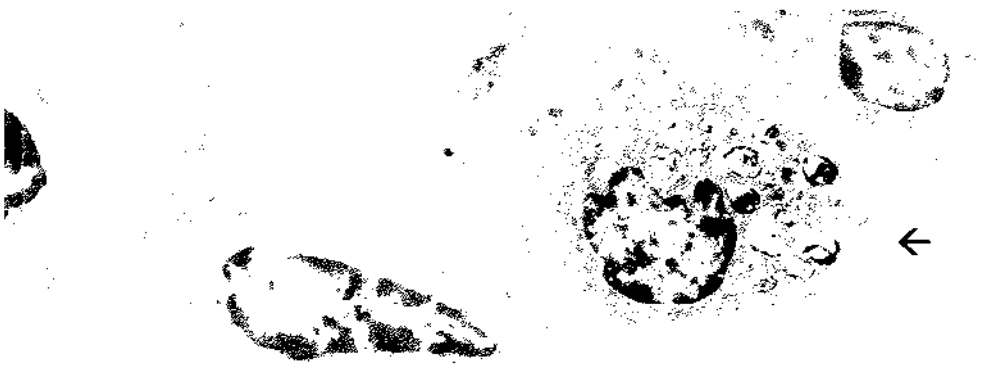

Figure 2.8 A type II cell (arrow) in this alveolus is easily identified by its lamellated bodies (cytosomes). TEM. Uranyl acetate-lead citrate. Magnification = 7800x.

LYMPHOID TISSUE

Mucosal associated lymphoid tissue (MALT) was first observed in the proximal airways in the lung of a possum aged 105 days, and was observed in most lung lobes in most possums from the age of 4 months onwards. This MALT consisted of discrete lymphoid aggregates, with central lymphoid follicles (germinal centres), lying in the submucosa of a large bronchiole (Figure 2.9), and ranged in size from 100-750 μm (mean = 250 μm). No lymphoepithelium was detected, nor

were there any specialised changes to the bronchiolar mucosa. Occasionally, discrete lymphoid aggregates were observed beneath the pleura, and were macroscopically evident as small 1-3 mm white nodules.

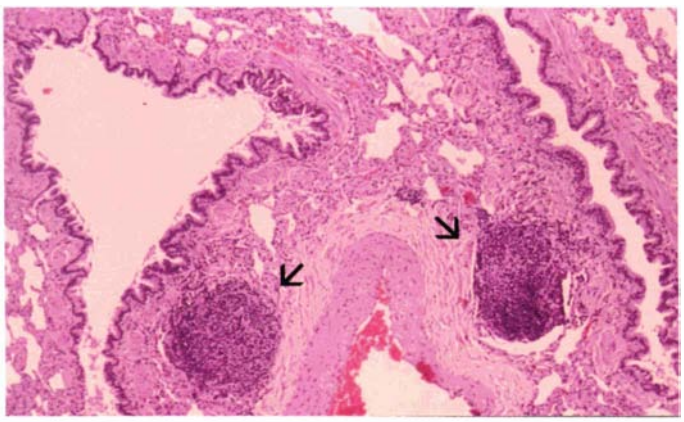

Figure 2.9 Discrete lymphoid nodules (arrows) were commonly observed adjacent to a major bronchiole in most possums' lungs, in most cases. H&E. Magnification = 50x.

2.4 DISCUSSION

The lung of the possum consists of six clearly defined lobes – left and right cranial and caudal, right middle, and right accessory. Sonntag (1921) originally described the lung of the brushtail possum as possessing two left and four right lobes. Later, in a generalised anatomical study of a marsupial, the mulgara (*Dasycercus cristicauda*), Jones (1949) named the four lobes of the right lung as the upper, middle, lower and azygos, and noted that the left lung showed only a partial subdivision into upper and lower lobes. Thus, the lobes of the lung of the brushtail possum are similar to these earlier descriptions in marsupials.

The lung parenchyma within each lobe of the brushtail possum was found to be supplied by major bronchioles, and there were no true bronchi within the lung. Thus no submucosal glands were present beyond the hilus of each lobe. Kennedy *et al.* (1978) reported a similar phenomenon in the lung of the hamster, where cartilage extends 1 mm after branching off from the primary bronchus, and there is a paucity of submucosal glands in the tracheobronchus. In the mouse, submucosal glands are restricted to the larynx and the most rostral part of the trachea (Pack *et al.*, 1981). Such features are further reflected in the composition of cells populating the bronchial mucosa. Goblet cells are a minor constituent of the tracheobronchial epithelium of the rat, mouse, hamster and rabbit (Plopper, 1983). However, Clara cells, which are usually confined to the bronchioles in most mammalian species, have been found in the bronchi of the hamster (Kennedy *et al.*, 1978), as well as the mouse and rabbit (Plopper, 1983). It is

interesting to note that laboratory animals such as the hamster, rat, mouse, guinea pig and rabbit are species which have high levels of chronic respiratory disease when kept in close confinement (Siegmund, 1979).

In the only available descriptions concerning bronchi in marsupials, Krause and Leeson (1973, 1975) described these structures of the American opossum as lined by ciliated and non-ciliated columnar epithelial cells. Scattered patches of ciliated cells predominated in the depths of folds of the bronchial mucosa. Non-ciliated cells, found singly and in groups, were described by these authors as containing granules in their apical cytoplasm. Clara (1937) originally described non-ciliated, non-mucous secretory cells of this type in the human and rabbit lung. Krause and Leeson (1973, 1975) observed few goblet cells, which were usually seen in the depths of the bronchial folds. A similar picture was observed in the current study of the bronchial epithelium of the brushtail possum, where Clara cells were present in moderately high numbers, and goblet cells were rare. Although it is not clear what is produced by Clara cells in marsupial lungs, it is likely that the low numbers of submucosal glands and goblet cells compared with eutharian mammals is compensated for by a predominance of non-ciliated, secretory Clara cells. To establish if a layer of mucus covers Clara cells, techniques involving fixation of the mucous layer with mucus-specific antiserum would facilitate its detection by conventional TEM (Lumsden *et al.*, 1994). Information produced by this method may demonstrate that the contribution to secretions from Clara cells is equivalent to the mucus secreted by goblet cells in the eutharian species, where Clara cells are confined to the bronchiolar airways. Thus, in eutharian species, the rôle of mucus-producing goblet cells may have taken on some of the function of Clara cells. Possums evolved some 50 million years ago (Flannery, 1994), and it is possible the difference between the lung of the possum and eutharian mammals may date back to that time.

The innate defence mechanisms of the lung include the mucociliary blanket, pulmonary surfactant, alveolar macrophages, MALT, immunoglobulins in the airway surface fluid, and antimicrobial peptides. Bienenstock *et al.* (1982) defined MALT as a lymphoepithelial nodule with a lymphofollicular structure intimately associated with an area of specially adapted mucosal epithelium. As was observed in the lung of the brushtail possum, lymphoepithelium is not always present covering MALT. This definition was considered too restrictive for the purposes of this study, and instead the broader definition, as proposed by Anderson *et al.* (1986), which includes all lymphoid tissue associated with the respiratory mucosae, whether or not covered by lymphoepithelium, was adopted. These authors noted that lymphoepithelium rarely overlies MALT in the normal, non-pneumonic (bovine) lung. However, lymphoid nodules, covered by normal respiratory epithelium, especially those observed in the lung of very

young possums, may represent an early stage in the development of MALT, which may subsequently mature as a result of further antigenic stimulation. Taking this into account, the lung of the brushtail possum appears to be adequately supplied with MALT in comparison with other species.

The possum's lung is also adequately supplied with alveolar macrophages. While no information exists on the presence or nature of immunoglobulins and antimicrobial peptides in the lung of brushtail possums, this study has suggested that there is a poorly developed mucociliary blanket in the possum, even at the level of bronchi. It is possible that this perceived deficiency is compensated for by the large numbers of Clara cells. However, clearance of secretions and foreign material at the bronchial level may be slower than that observed in other species. More work is required on this aspect, including *in vivo* and *in vitro* experiments, such as inoculating organisms like *Staphylococcus* spp into the lung, and measuring bacterial clearance in the trachea compared with the lung at different time intervals.

Kahwa *et al.* (1997) consider that information on the morphological features and cell differentiation of the distal airways is essential before pathological lesions due to disease processes can be interpreted. Although morphologically the brushtail possum has a poor mucociliary defence, it would be unwise to extrapolate this to infer that the lung of the possum is more susceptible to infection with *M. bovis* than other species, without further pathophysiological investigation. However, the lack of true bronchi after the hilus of each lung lobe, coupled with a poorly developed mucociliary system, may render the possum lung less capable of clearance of particles larger than droplet nuclei, which would normally be cleared to the orophranynx and swallowed.

CHAPTER 3. THE PATHOLOGY OF NATURALLY OCCURRING *MYCOBACTERIUM BOVIS* INFECTION IN BRUSHTAIL POSSUMS

3.1 INTRODUCTION

The first two documented but unconfirmed cases of tuberculosis in possums (*Trichosurus* spp.) occurred in captive possums outside of Australasia (Moore, 1903; Scott, 1928). It was not until 1967 that tuberculosis was recorded in a wild possum (Ekdahl *et al.*, 1970), and it was also the first time that the disease was confirmed by both culture and histopathology. The possum, which had discharging sinuses, was trapped near Westport, New Zealand. It was one of 20 from a total of 25 animals which had similar lesions. Another possum from the same area died of advanced pulmonary tuberculosis. From this time onwards, the generalised nature of the disease, the suppuration of lesions, and the vast numbers of organisms in affected tissues (estimated by Smith (1972) to be in the vicinity of 5×10^9 per 1 gram of tissue) became recognised as the hallmarks of tuberculosis in this species.

The discovery of tuberculosis in possums in several areas of New Zealand where the level of the disease in cattle was high and intractable (Cook, 1975) prompted epidemiological, field, and experimental studies. These studies helped confirm the possum's rôle as a wildlife reservoir host of the disease (Coleman, 1975; Cook, 1975; Cook and Coleman, 1975; Lake, 1975; Stockdale, 1975; Davidson, 1976; Julian, 1981). Later, DNA restriction endonuclease analysis facilitated the typing of *M. bovis* isolates to identify sources of infection in specific areas (Collins *et al.*, 1986).

In the four field studies of tuberculosis in possums undertaken in New Zealand to date (Cook, 1975; Cook and Coleman, 1975; Lake, 1975; Hickling *et al.*, 1991) only limited use of laboratory diagnostic techniques was made and only a small range of tissues were examined. Due to the lack of standardisation of techniques, variability in the skills of field staff in conducting post mortem examinations and recognising tuberculous lesions, difficulties in collecting samples aseptically, lack of samples from possums with no visible lesions, and differences with regard to diligence in recording data, the absolute rate of infection within a possum population was likely to have been underestimated. However, these studies provided an estimate of the percentage of possums affected in a given area, and data on how far into the

bush tuberculous possums could be found. They also provided information on the nature and distribution of macroscopic lesions.

Lake (1975) gave the first pathological description of lesions in the natural disease in possums. He described pulmonary lesions as solitary nodular areas of consolidation, which occasionally coalesced, most of which contained centres with variable amounts of lime green inspissated pus. Affected lymph nodes were composed almost entirely of pus. Histologically, pulmonary nodular consolidation was composed of granulomatous tissue, with no distinctive fibrous capsule and centrally there were varying amounts of amorphous eosinophilic debris. The inflammatory infiltrate included lymphocytes, plasma cells, an occasional eosinophil, macrophages, giant cells, and many neutrophils, which were most concentrated around areas of caseation. Both free and intracellular acid fast organisms (AFOs) were often present in large numbers.

In their experimental infections of possums with *M. bovis*, Pfeffer *et al.* (1994) reported a wider distribution of microscopic than macroscopic lesions, as well as the involvement of major organs and most lymph nodes. They commented that it was unknown if their findings reflected the natural disease, as extensive examination of wild possums for microscopic lesions had not been reported.

The results presented here are based on material obtained in various field studies of possum populations in which tuberculosis was present (Cooke *et al.*, 1995). These studies provided an opportunity for a detailed description of the nature and distribution of lesions in the naturally occurring disease, making use of both microbiology and histopathology on an extensive range of tissues and sites. The study was undertaken to provide information to support or refute theories on the pathogenesis of the disease generated from other avenues of investigation, as well as to produce new hypotheses.

3.2 MATERIALS AND METHODS

3.2.1 Source of possums

Tuberculous and terminally ill possums were sourced from seven cross-sectional studies in Westland (Coleman *et al.*, 1993, 1994a, 1994b, 1996; Coleman and Cooke, 1995, 1997, 2000), a

cross-sectional study at Hauhungaroa and another at Tinui, and from cross-sectional and longitudinal studies near Castlepoint (Pfeiffer and Morris, 1991).

Possums from all sites except for Castlepoint and Westland in December 1992 were collected using Victor 1½ leg-hold traps, with a lure prepared from a mix of wholemeal and standard flour, icing sugar, and cinnamon or banana essence and set on lines. Possums from Castlepoint were collected using cage traps baited with apple. Each day, all captured possums were humanely killed and tagged, and the trap site recorded, before the carcases were removed to a central necropsy site. In December 1992, possums were poisoned in Westland using cyanide paste, and carcases were collected over the next 3 days.

The terminally ill possums were considered as a separate group, based on their behaviour immediately prior to their death, or on the situation in which they were found dead. They were placed in this group if they were seen out in daylight exhibiting aberrant behaviour, or were found dead with generalised tuberculosis in unusual or open sites. This distinct group of possums was used for collection of data illustrative of the end stage of the disease.

3.2.2 Necropsy and data recording procedures

The sex, maturity (determined from the size of the gonads and presence of a pouch), colour, trap site, body weight and length, and reproductive status of each carcase were noted (Appendix VI). Body condition was determined from the weight of the excised mesenteric fat depot (Bamford. 1970). For ageing, either the right mandible was removed and the second and third molars extracted for assessment of cementum annuli (Pekelharing, 1970), or the pattern of tooth wear on the upper left first molar was scored (Winter, 1980). In the few cases where age was not assessed by either of these methods, possums were classified either as immature or mature When a joey was present, its sex and head length were recorded.

All major lymph nodes, thoracic and abdominal organs, and mammary glands were palpated and examined, and the presence of palpable and macroscopic lesions suspected as being tuberculous were noted (Appendix VI). Possums were classified as macroscopically tuberculous if they had soft, swollen lymph nodes or nodules in visceral organs that measured 3 mm or more in diameter. Possums were classified as "suspicious" if they had enlarged, firm lymph nodes or had nodules in visceral organs that measured less than 3 mm in diameter. Macroscopic lesions in lymph nodes were classified as enlarged, if the nodes were soft and swollen, and caseous if the nodes contained cream or lime-green, fluid or semi-fluid, contents.

Peripheral lymph nodes were described as pointing if they had the appearance of an abscess about to burst, and discharging if they had burst and the contents were discharging to the exterior through sinuses.

3.2.3 Collection of samples for histopathology

Tissues from up to 40 separate body sites were collected for histopathological examination from possums classified as macroscopically affected or suspicious, as well as from a random selection of possums with no macroscopically visible lesions (NVL). The 40 body sites were the left and right deep and superficial axillary and cervical lymph nodes; left and right inguinal, mandibular, parotid and anterior mediastinal lymph nodes; mesenteric, gastric and hepatic lymph nodes; left and right palatine tonsils, kidneys, and adrenal and mammary glands; left and right cranial and caudal lung lobes; right middle and accessory lung lobes; liver, spleen, bone marrow, duodenum, ileum, colon, and thymus.

The anterior mediastinal lymph nodes were removed attached to connective tissue around the lung. All other lymph nodes and mammary glands were stapled on to waterproof sheets of paper (Appendix VII). By removing the kidneys with the adrenal glands attached, it was possible to easily identify left from right. The left adrenal gland lies on the concave side of the left kidney, whereas the right adrenal gland lies in a small fossa in the liver at the cranial pole of the right kidney.

A 2-3 cm section of the duodenum was collected just cranial to the entrance of the pancreatic duct into the intestine, the distal 2-3 cm of the terminal ileum was sectioned just prior to its entry into the caecum, and the colon was sectioned 10-15 cm cranial to the rectum. If no macroscopic lesions were evident, random sections of spleen and liver were taken. Bone marrow was collected after breaking off the proximal 3-4 cm of the left humerus.

3.2.4 Selection of fixed tissues for histopathological examination

After fixation in 10% neutral buffered formalin for at least 1 week, all lymph nodes were measured and the nature, size and distribution of any macroscopic lesions in these and in other tissues were recorded, complementing data recorded earlier in the field (Appendix VI). Any macroscopic lesions evident in lung, liver, kidneys or spleen were embedded in paraffin blocks for histopathological examination. If no macroscopic lesions were evident in these organs,

samples were randomly selected for histology. In the case of the lung, a standard technique was adopted to optimise the chances of finding lesions in each of the six lobes of the lung. This entailed taking a cross-section from the middle of each of the lobes. All other tissues (lymph nodes, palatine tonsils, adrenal and mammary glands, bone marrow and intestine) were embedded in paraffin, without prior macroscopic incision, and routinely processed for the production of 4 μm thick sections stained by haematoxylin and eosin (H&E) and Ziehl-Neelsen[1] (ZN) stains.

Possums were only classified as histopathologically positive if the lesions had a characteristic morphology, and at least one lesion contained AFOs.

3.2.5 Selection of specimens for bacteriology

Where possums were classified as macroscopically affected or suspicious, representative samples of macroscopic lesions (usually a lymph node, but occasionally aspirated content or sterile swabs in transport medium) were collected aseptically and stored in a chilled state until they were frozen at the end of each day. These specimens were sent to AgResearch, Wallaceville, for culture of mycobacteria, according to the methods of Buddle *et al.* (1994). Exceptionally, in December 1992, no specimens were collected for culture. No cultural work was performed on six NVL possums sampled for histopathological examination. Pooled nodes were collected from randomly selected NVL animals, which did not include those six sampled for histopathological examination only.

3.2.6 Specimens for electron microscopy

Samples of fresh tuberculous lung and affected lymph nodes were fixed in 3% gluteraldehyde. Together with other samples from formalin-fixed tissues, these tissues were post-fixed in 1% osmium tetroxide in phosphate buffered saline (pH 7.4) for 1 hour and embedded in epoxy resin (Procure 812, Probing and Structure, Thuringowa, Queensland, Australia). Thin sections were cut and mounted on copper grids before staining with uranyl acetate and lead citrate, and the grids examined by transmission electron microscopy (TEM) (Philips 201c TEM).

[1]Culling CFA, Allison RT, Barr WT (eds). Cellular Pathology Technique. 4th Edtn. P 336. Butterworths, London, 1985.

3.2.7 Statistical analysis

The Chi-squared (χ^2) test was used to assess the statistical significance of tuberculous possums which were males compared with those which were females; lesions present in the respiratory tract but absent in superficial lymph nodes (and vice versa); the association between possums with discharging sinuses in superficial lymph nodes and the number of lobes of the lung affected macroscopically; and for comparing differences in the nature and distribution of lesions in non-terminally ill and terminally ill possums. The statistical significance of total lesions at five body sites was tested by applying the McNemar's test, to see whether there was evidence that, if an animal only had one site affected it was significantly more likely to be that site than another, or whether the differences could have occurred by chance. All statistical analyses were conducted with the aid of a statistical computer software package (Statistix, Analytical Software Co., La Jolla, California).

3.3 RESULTS

3.3.1 Prevalence of tuberculosis

A total of 1659 possums were trapped from the 10 cross-sectional studies (Table 3.1). Because longitudinal studies are not point prevalence studies, no data have been presented from them on the number of normal and tuberculous possums, nor on the prevalence of tuberculosis according to population and sex. Based on the isolation of *M. bovis*, 99 possums derived from cross-sectional studies and all animals derived from the longitudinal study were confirmed as tuberculous. A further 30 possums from cross-sectional studies were confirmed as being tuberculous by histopathology, including eight NVL animals. One hundred and fifteen of the total 129 tuberculous possums sourced from cross-sectional studies were included in this study, together with a further 22 tuberculous possums (most of them terminally ill) derived from the longitudinal study at Castlepoint. They were divided into one group of 117 non-terminally ill possums, and a second group of 20 terminally ill possums. The exclusion of 14 tuberculous animals from the 129 sourced from cross-sectional studies was due to 12 NVL possums diagnosed as being tuberculous by culture of pooled lymph nodes, hence no lesion distribution was available; the diagnosis by histopathological examination of a section of liver from a possum from Westland in December 1992; and the isolation of *M. bovis* from macroscopic lesions in the autolysing carcase of a possum from Westland in August 1993. As that carcase was partially scavenged, its sex could not be determined.

Table 3.1 Source of tuberculous and terminally ill possums from field studies

Survey	No. Necrop	No. Male	No. Female	Total TB[a]	TB Male[b]	TB Female[b]	No. in Study
FF Aug 1992	68	35	33	41 (60%)	26 (63%)	15 (37%)	38
FF Dec 1992	119	46	73	24 (20%)	10 (42%)	14 (58%)	23
FF Aug 1993	54[c]	29	24	9[c] (17%)	7 (88%)	1 (12%)	6
FF Aug 1994	77	45	32	7 (9%)	4 (57%)	3 (43%)	7
FF Aug 1995	98	52	46	4 (4%)	4 (100%)	0	4
FF Aug 1996	130	72	58	4 (3%)	3 (75%)	1 (25%)	2
FF Dec 1999	143	71	72	7 (5%)	5 (71%)	2 (29%)	7[d]
HH Apr 1994	232	142	90	2 (0.9%)	1 (50%)	1 (50%)	2
Tinui	310[c]	145	164	5 (1.6%)	3 (60%)	2 (40%)	3
CP kout 1994	428	226	202	26 (6%)	14 (54%)	12 (46%)	23[d]
TOTAL	1659	863	794	129 (7.8%)	77 (60%)	51 (40%)	115 + 22[e]

Necrop = necropsied; TB = tuberculous; FF = Flagstaff Flat; HH = Hauhungaroa; CP kout = Castlepoint killout (Sept-Oct, 1994)
[a]Percentage figures denote point prevalence of tuberculosis
[b]Percentage figures represent the proportion of tuberculous possums which were either male or female
[c]Includes one possum of unknown sex
[d]Includes terminally ill possum(s)
[e]22 possums were sourced from the longitudinal study at Castlepoint

Although about 8% more male than female possums were captured, proportionately more tuberculous males (60%, range 42% to 100%) than tuberculous females (40%, range 0 to 58%) were obtained. On only one occasion, viz. Flagstaff Flat in December 1992, were more tuberculous females than males collected. The percentage of male and female tuberculous possums is equal in two instances (Flagstaff Flat in August 1994, and the Castlepoint killout in 1994), and the proportion of males exceeds the proportion of females in all but one instance (Hauhungaroa, when 1.1% of females were tuberculous compared with 0.7% of males).

3.3.2 Distribution of lesions

TUBERCULOUS POSSUMS

Macroscopic lesions

Macroscopically visible lesions of tuberculosis were detected in 109 possums, and confirmed by both mycobacterial culture and histopathology in 88 of these. The diagnosis of tuberculosis was made in the remaining 21 possums with macroscopic lesions, and eight NVL possums by histopathological examination showing AFOs in typical lesions. The eight possums free of macroscopic lesions were: B6104, B6113, G1097, H3126, H3132, K0973, A42865, D47725. The distribution of macroscopic and microscopic lesions in the 117 tuberculous possums is presented in Appendix VIII. Significantly more of the tuberculous possums were male (73/117

or 62%) than were female (44/117 or 38%) (χ^2 = 7.19, p<0.01). Affected possums ranged in age from 6-month-old back-riders to 9 years (average of 2.9 years). The age in years was not determined in four (3%) possums.

The number of macroscopically visible lesions per possum varied from one to 25, with a mean of 6.7 (Figure 3.1). The most common sites for lesions, in order of abundance, were the individual lobes of the lung, the liver, and the left superficial axillary lymph node (Figure 3.2).

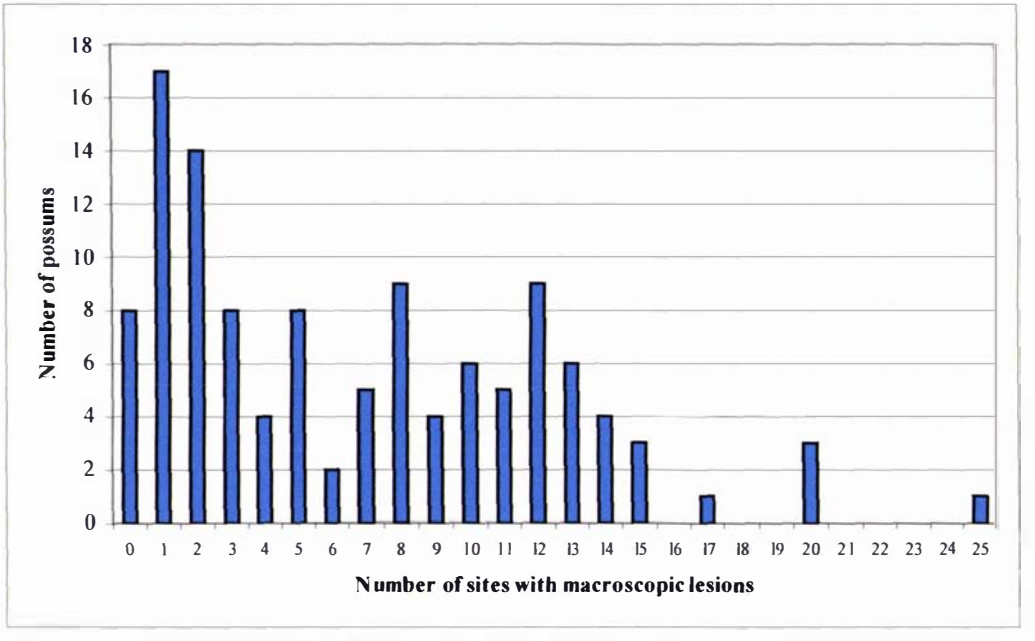

Figure 3.1 Frequency of number of sites containing macroscopic lesions per individual in 117 tuberculous possums.

In order to assist in the interpretation of the data, the lesion sites were grouped into five major body sites, according to their relatedness. These were "superficial", comprising the inguinal and deep and superficial axillary lymph nodes; "head and neck", consisting of the palatine tonsils, and mandibular, parotid, and deep and superficial cervical lymph nodes; "respiratory", representing all six lobes of the lung and the anterior mediastinal lymph nodes; "gastrointestinal", made up of the mesenteric, gastric and hepatic lymph nodes, liver, duodenum, ileum and colon; and "other sites", including the kidneys, adrenal and mammary glands, spleen, bone marrow and thymus. When lesion distribution was assessed according to these five body sites, macroscopic lesions were most common in the superficial lymph nodes (75%), followed by the respiratory tract (69%), and the gastrointestinal tract (GIT) (53%) (Figure 3.3 and Appendix IX).

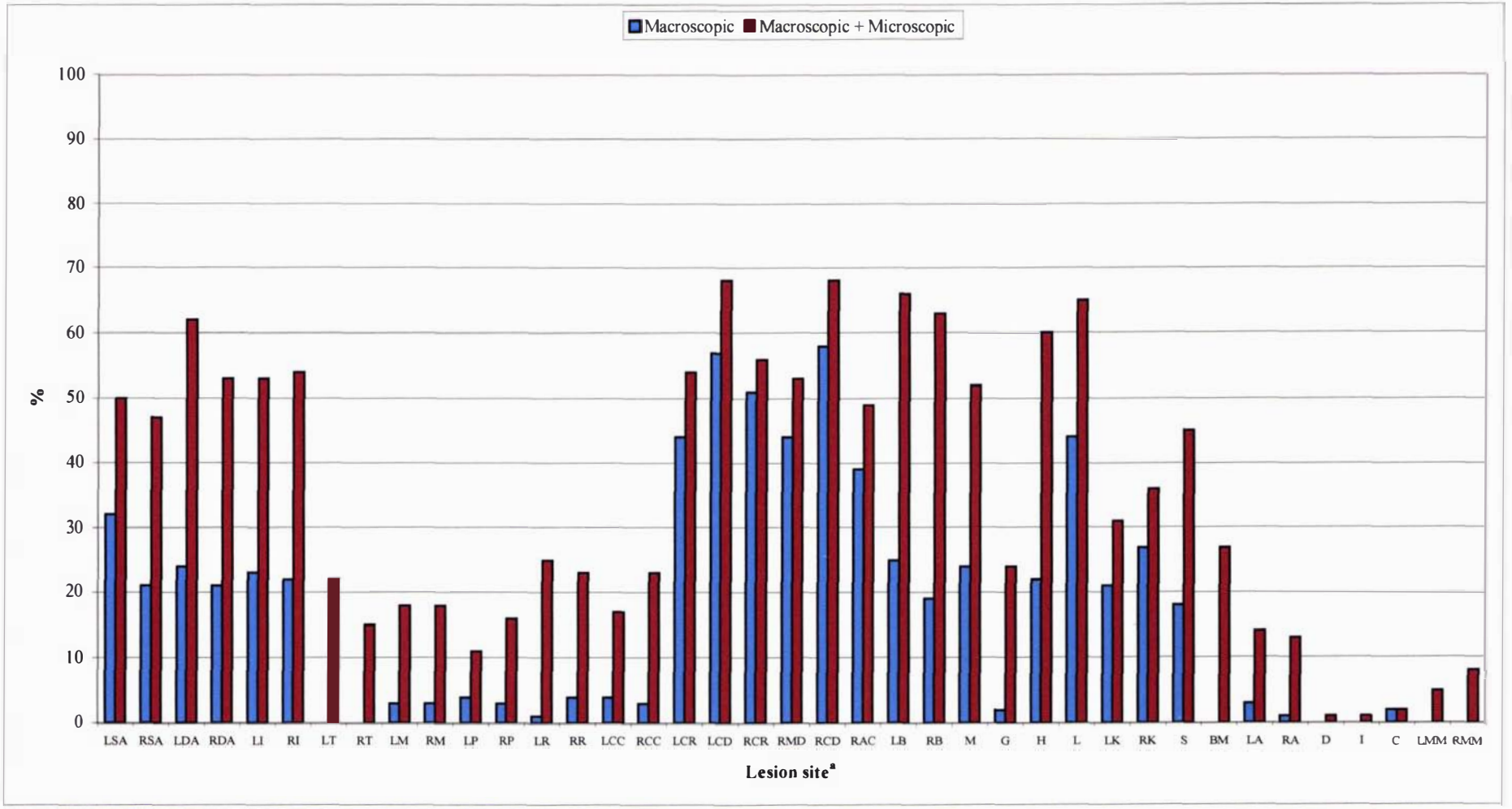

Figure 3.2 Distribution of macroscopic and microscopic tuberculous lesions in 117 possums derived from field studies.

([a]LSA/RSA = left/right superficial axillary ln; LDA/RDA = left/right deep axillary ln; LI/RI = left/right inguinal ln; LT/RT = left/right palatine tonsil; LM/RM = left/right mandibular ln; LP/RP = left/right parotid ln; LR/RR = left/right deep cervical ln; LCC/RCC = left/right superficial cervical ln; LCR/RCR = left/right cranial lung lobe; LCD/RCD = left/right caudal lung lobe; RMD = right middle lung lobe: RAC = right accessory lung lobe; LB/RB = left/right anterior mediastinal ln; M/G/H = mesenteric/gastric/hepatic ln; L = liver; LK/RK = left/right kidney; S = spleen; BM = bone marrow; LA/RA = left/right adrenal gland; D = duodenum; I = ileum; C = colon; LMM/RMM = left/right mammary gland; ln = lymph node)

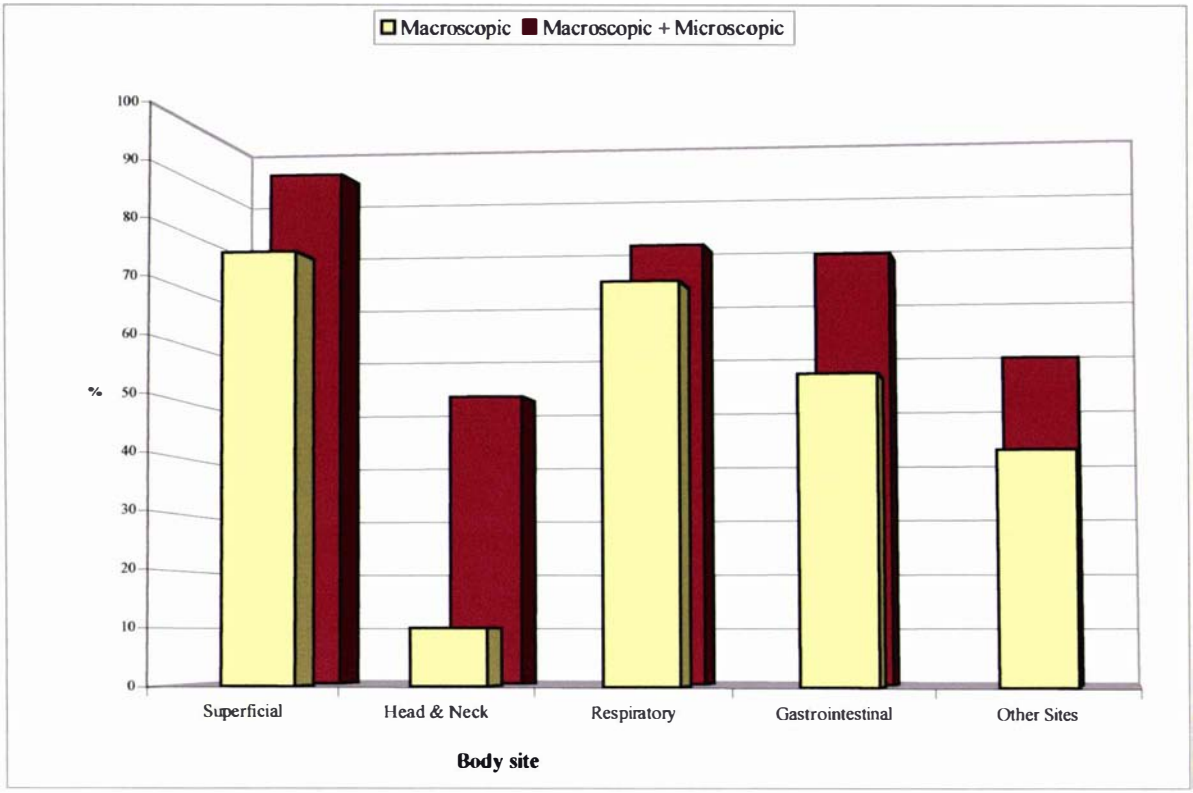

Figure 3.3 Distribution of macroscopic and microscopic tuberculous lesions at five body sites in 117 possums.

Macroscopic lesions recorded in superficial lymph nodes and not in the respiratory tract occurred on 27 (23%) occasions, compared with 20 (17%) incidents in which macroscopic lung lesions were seen but no macroscopic lesions were present in superficial lymph nodes; however this was not statistically significant (χ^2 = 0.001, p<1.0). A total of 167 individual superficial lymph nodes were affected, 93 (56%) of which were on the left side of the body.

No macroscopic lesions were detected in the thymus, palatine tonsils, bone marrow, mammary glands, duodenum and ileum.

Macroscopic lesions considered as possibly tuberculous but subsequently attributed to other causes were detected on 54 occasions in 35 possums. These were most frequently encountered in the liver, kidney, lung and lymph nodes (Table 3.2). Blood was frequently aspirated terminally into the lungs during humane killing of possums caught in leg-hold traps. Once the cadavers cooled, blood clots related to this phenomenon were palpated as small, firm, lesions (Cooke *et al.*, 1995). Adiaspiromycosis could not be differentiated macroscopically from 1-2 mm tuberculous lesions when the two entities occurred together. Subpleural lipid plaques and lymphoid nodules less than 5 mm in diameter could not be macroscopically distinguished from small tuberculous lesions, visually or by palpation, in a cooled carcase. In almost all

possums, about six to ten small (about 1 mm in diameter) firm nodules were palpable in the caudal tip of the left caudal lung lobe. Histologically, these were identified as fibrous thickenings of alveolar septa. The cause of these lesions could not be determined. Small tuberculous renal lesions could not be distinguished macroscopically from either multi-focal chronic interstitial nephritis or nephrosis. Hepatic lesions provisionally classified as tuberculous were in some cases found to be due to other causes such as focal fatty change. All macroscopic lesions seen only in either the kidney or liver, and in no other sites, were subsequently found to be due to causes other than tuberculosis. In lymph nodes, the most common finding other than tuberculosis was enlargement due to individual variation in lymph node size.

Table 3.2 Frequency of differential diagnoses at tissue sites in 117 tuberculous possums

Site	Number with other diagnoses	Other diagnoses[a]
Lymph nodes	15 (<1%)	1
Lung	18 (2.6%)	1, 2 & 3, 4
Liver	8 (7%)	1, 3, 5
Kidney	12 (5%)	6, 1 & 7, 5
Spleen	1 (<1%)	8

[a]Other diagnoses, in descending order of frequency of occurrence, were:
1 = no significant findings; 2 = adiaspiromycosis; 3 = lipid; 4 = foreign body;
5 = parasite; 6 = nephrosis; 7 = interstitial nephritis; 8 = amyloid

Microscopic lesions

Histopathological examination greatly increased the detection of lesions, especially in lymphoid tissues of the head and neck (Figure 3.2 and Appendix VIII). The number of macroscopic plus microscopic lesions varied from one to 34 per possum, with a mean of 13.1 (Figure 3.4). The most common sites for lesions were the six lung lobes and their associated lymph nodes, the liver and its associated (hepatic) lymph node, and the superficial lymph nodes. When grouped according to the five major body sites, the distribution of lesions was statistically significant, as 93% of tuberculous possums had lesions in superficial lymph nodes, compared with 79% in the respiratory tract (McNemar's test = 8.0, $p = 0.005$) (Figure 3.3 and Appendix IX).

Macroscopic plus microscopic (total) lesions recorded in superficial lymph nodes and not in the respiratory tract occurred on 24 (21%) occasions, compared with eight (7%) incidents in which total lung lesions were seen but no lesions were present in superficial lymph nodes. This was not statistically significant ($\chi^2 = 0.61$, $p<0.5$). There was a significant difference in the frequency of total lesions in the left plus right inguinal lymph nodes between tuberculous males and tuberculous females ($\chi^2 = 4.36$, $p = 0.04$) (see Addendum opposite).

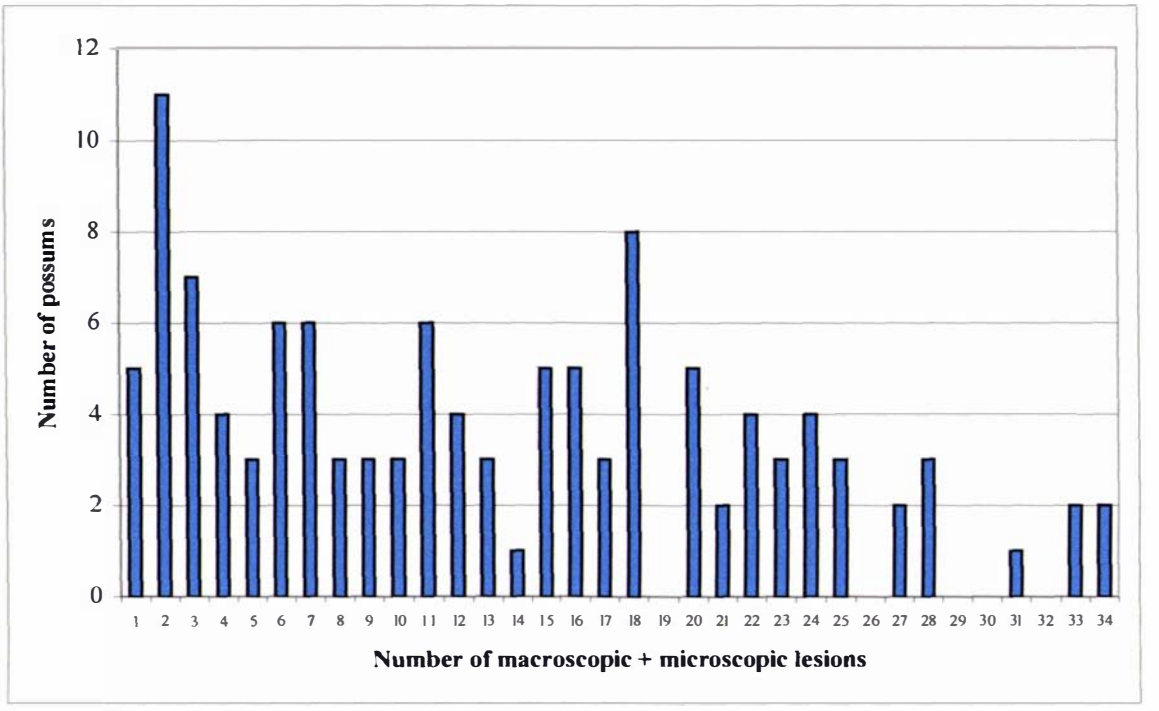

Figure 3.4 Frequency of number of sites containing macroscopic and microscopic lesions per individual in 117 tuberculous possums.

TERMINALLY ILL POSSUMS

Macroscopic lesions

Twelve (60%) of the terminally ill possums were male, and 8 (40%) were female, but too few possums were in this group for this to be statistically significant. However, this proportion was very similar to that recorded for the non-terminally ill possums (see Page 54). Affected possums ranged in age from 7 months to 7 years, although age was not determined for 50% of the possums.

The number of macroscopic lesions per possum varied from 8 to 24, with a mean of 13.2. All of the terminally ill possums had lesions in their lungs. The next most common site for lesions outside the respiratory tract was the mesenteric lymph nodes (Figure 3.5 and Appendix X). When lesion distribution was grouped according to the five major body sites, all possums had lesions in the respiratory tract, and the next most common site for lesions was the GIT (Figure 3.6 and Appendix XI).

No macroscopic lesions were detected in the thymus, palatine tonsils, left mammary gland, ileum, and right superficial cervical lymph node.

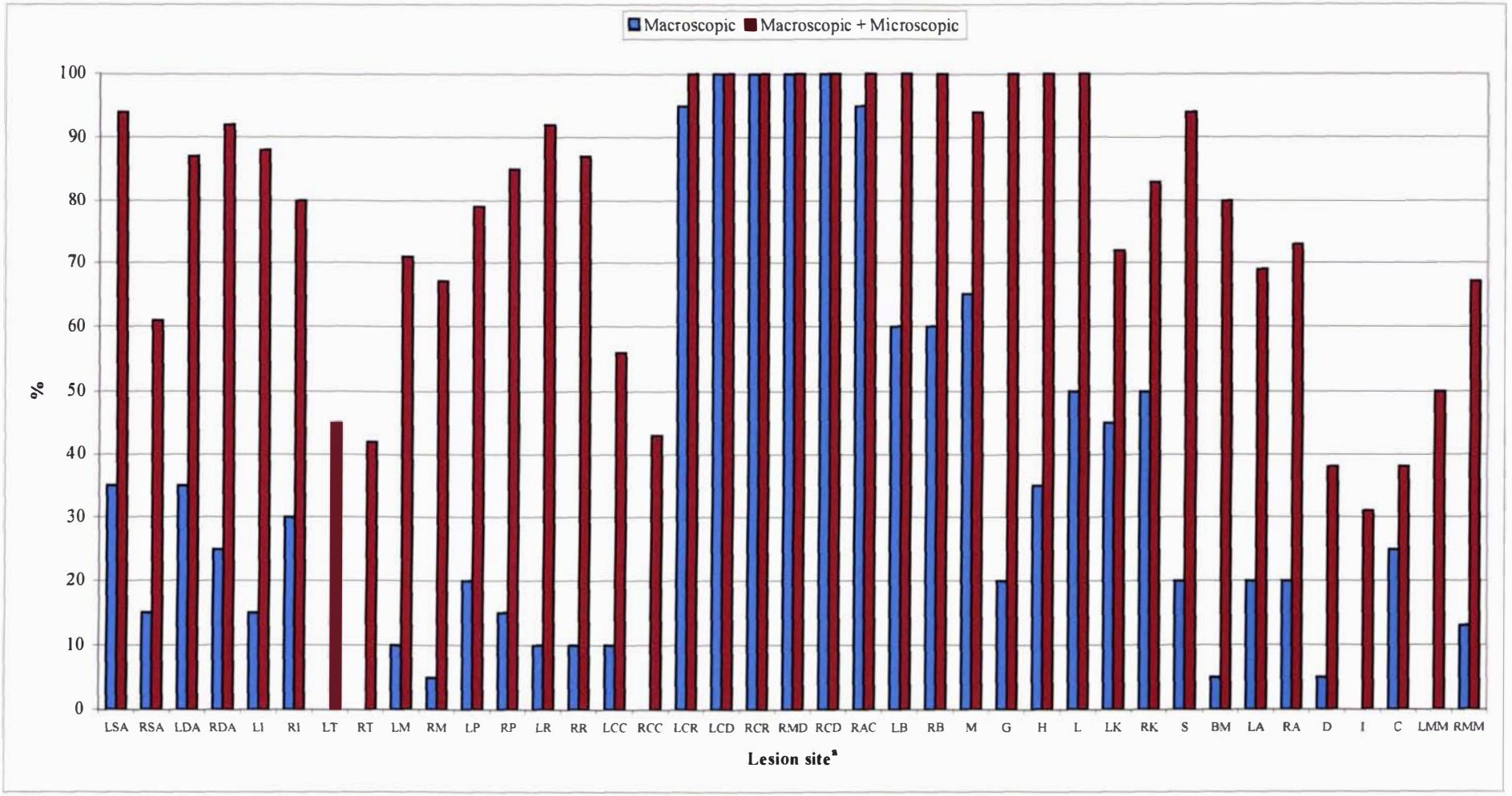

Figure 3.5 Distribution of macroscopic and microscopic lesions in 20 terminally ill possums derived from field studies.

([a]LSA/RSA = left/right superficial axillary ln; LDA/RDA = left/right deep axillary ln; LI/RI = left/right inguinal ln; LT/RT = left/right palatine tonsil; LM/RM = left/right mandibular ln; LP/RP = left/right parotid ln; LR/RR = left/right deep cervical ln; LCC/RCC = left/right superficial cervical ln; LCR/RCR = left/right cranial lung lobe; LCD/RCD = left/right caudal lung lobe; RMD = right middle lung lobe: RAC = right accessory lung lobe; LB/RB = left/right anterior mediastinal ln; M/G/H = mesenteric/gastric/hepatic ln; L = liver; LK/RK = left/right kidney; S = spleen; BM = bone marrow; LA/RA = left/right adrenal gland; D = duodenum; I = ileum; C = colon; LMM/RMM = left/right mammary gland; ln = lymph node)

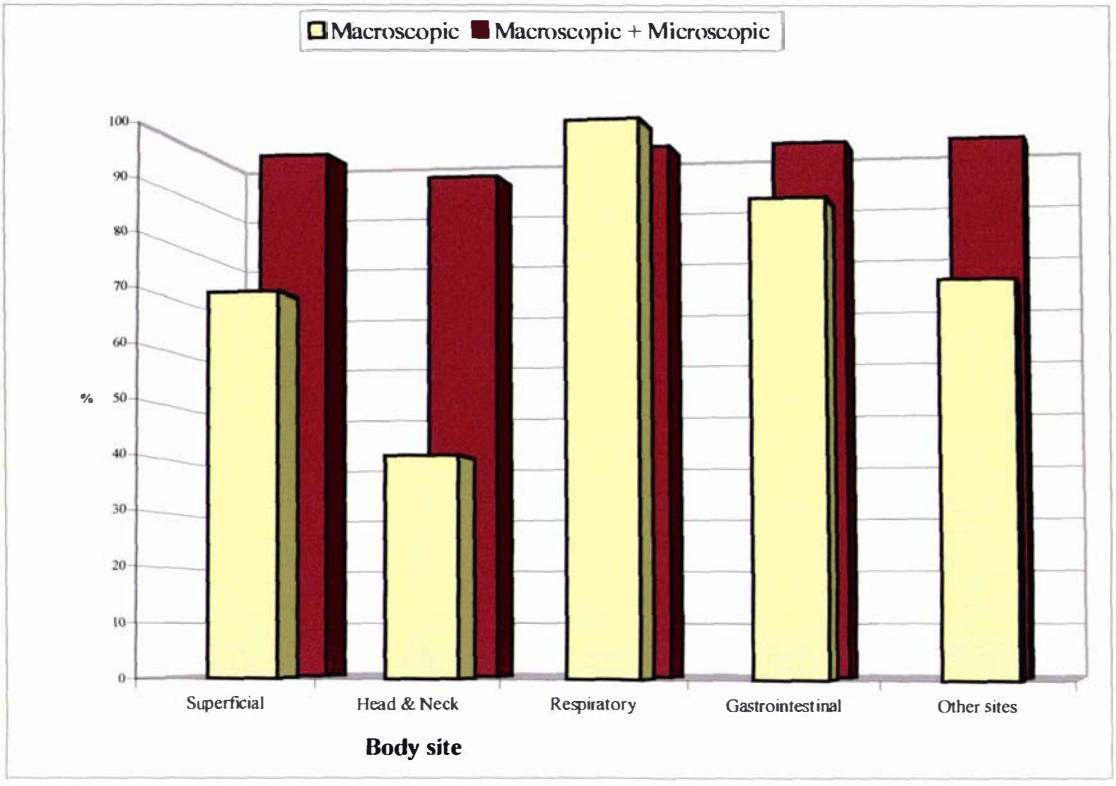

Figure 3.6 Distribution of macroscopic and microscopic lesions at five body sites in 20 terminally ill possums.

Microscopic lesions

When compared with the frequency of macroscopic lesions, histopathological examination increased the detection of (total) lesions by almost 100%, especially in lymphoid tissues of the head and neck (Figure 3.5 and Appendix X). The number of macroscopic plus microscopic lesions per possum varied from 13 to 38, with a mean of 25.1. The most common sites for lesions were the six lung lobes and their associated lymph nodes, the liver and its associated (hepatic) lymph node, and the gastric lymph nodes. When grouped according to the five major body sites, the distribution of lesions was more uniform, as all possums had lesions in superficial lymph nodes, respiratory tract, GIT, and other sites, and only 5% of possums did not have lesions in the lymphoid tissue of the head and neck (Figure 3.6 and Appendix XI).

Tuberculous lesions detected incidentally at sites other than the 40 routinely examined included granulomatous foci in the soft palate, gastric mucosa, and myocardium.

3.3.3 Nature of lesions

TUBERCULOUS POSSUMS

Macroscopic lesions

Palpably or visibly enlarged, caseous, pointing, or discharging superficial lymph nodes were recorded on 167 occasions, in 88 (75%) possums, 59 (67%) of which were males (Table 3.3). Discharging sinuses were observed in 35 (30%) possums, 23 (66%) of which were males (Figure 3.7). All but three of these possums had generalised tuberculosis (defined for this purpose as lesions at two or more body sites).

Table 3.3 Nature and distribution of macroscopic lesions in superficial lymph nodes of 88 affected possums

Lymph node[a]	Palpable/Enlarged	Caseous	Pointing	Discharging	Total
L super axillary	5	15	4	14	38
R super axillary	5	12	2	5	24
L deep axillary	11	17	0	0	28
R deep axillary	9	14	0	1	24
L inguinal	7	13	1	6	27
R inguinal	5	9	2	10	26

[a]L = left; super = superficial; R = right

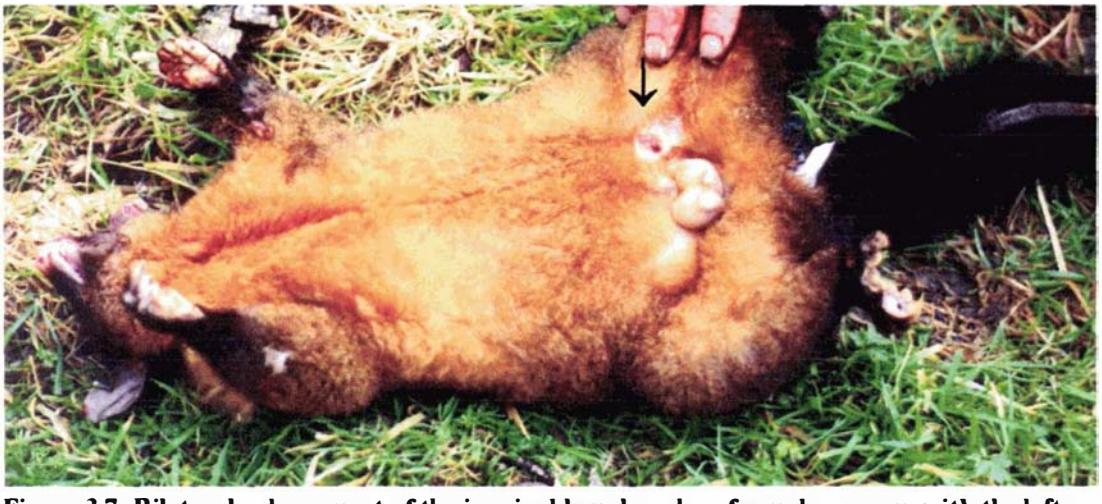

Figure 3.7 Bilateral enlargement of the inguinal lymph nodes of a male possum, with the left node (arrow) discharging its contents via a sinus in the skin.

There was a statistically significant association (χ^2 = 7.2, $p<0.01$) between possums with discharging sinuses in superficial lymph nodes and these animals having all six lobes of the lung affected (Table 3.4).

Table 3.4 Correlation between possums with discharging sinuses in superficial lymph nodes and the presence of macroscopic lesions in 0-6 lobes of the lung

	+ Macroscopic lesions present in lung lobes:						
	0 lobes	1 lobe	2 lobes	3 lobes	4 lobes	5 lobes	6 lobes
Possums WITH sinuses (n = 35)	3 (8%)	1 (3%)	1 (3%)	2 (6%)	1 (3%)	1 (3%)	26 (74%)
Possums WITHOUT sinuses (n = 53)	24 (45%)	5 (9%)	2 (4%)	4 (8%)	6 (11%)	2 (4%)	10 (19%)

Lesions at lymphocentres (a collective term used by Hopwood (1980) to define a group of lymph nodes at a particular site) sometimes affected only one node, but more commonly incorporated all or most of the other nodes in the lymphocentre. On incision, enlarged nodes were usually pale, turgid and occasionally oedematous. A very few nodes contained small discrete green or cream-coloured foci but in most the lesions were extensively suppurative (Figure 3.8). The largest affected lymph node was a caseous left deep axillary node measuring 60 x 50 mm. The normal range in size of this node, assessed in 56 non-tuberculous possums, was between 2 and 22 mm in length, and 2 to 12 mm wide. Lymph nodes which were severely distended sometimes had a thickened fibrous capsule. Their contents ranged from bright lime green, liquefactive material, common in superficial lymph nodes, to cream, firm caseous material more in lesions especially seen in internal lymph nodes. Macroscopic evidence of mineralisation of lymph nodes was not observed at necropsy.

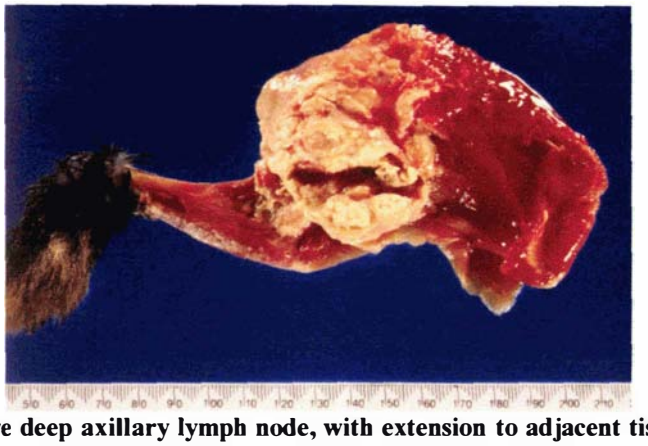

Figure 3.8 Suppurative deep axillary lymph node, with extension to adjacent tissues, from a tuberculous possum.

Of 80 (68%) possums which had macroscopic lesions in the lung, 52 (65%) were males. Pulmonary nodular lesions, including miliary lesions of 1-3 mm diameter distributed throughout all lung lobes, ranged from 1 to 60 mm in diameter (Figure 3.9). Although discrete nodular lesions were the most common lung lesion observed, large irregularly shaped areas (>30 mm in diameter) of firm, grey consolidation were observed in 19 (24%) possums with macroscopic lung lesions, and occurred in conjunction with nodular lesions. Fibrous pleural adhesions

between affected lung lobes or between the pulmonary pleura, pericardium or thoracic wall were seen in seven (9%) cases of pulmonary tuberculosis, but were not always seen in association with consolidation. Although the left and right caudal lung lobes were affected more often than other lobes, the density of lesions was proportionately equal after allowing for the greater volume of these lobes.

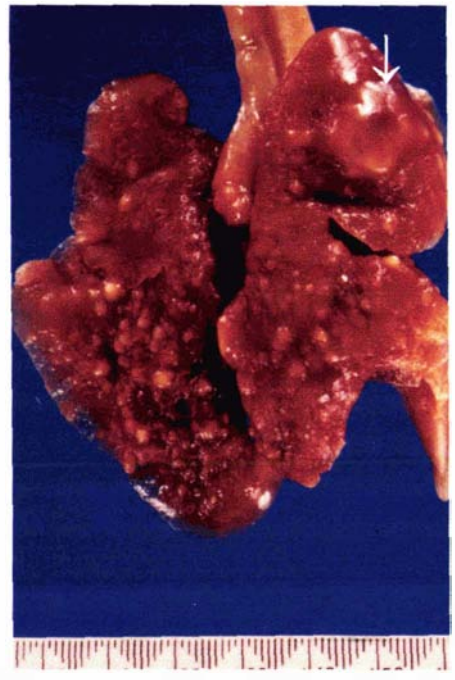

Figure 3.9 Fixed lung containing numerous small nodules and one large nodule in the right cranial lobe (arrow).

In other tissues and organs, lesions ranged from firm, cream to white, nodules, smaller than 1 mm diameter, through to large, cream-coloured soft caseous lesions. Hepatic and renal lesions were generally small, with most 1 to 2 mm in diameter. The largest liver lesion was 11 mm in diameter and lesions in the liver were randomly distributed throughout the parenchyma. Renal lesions primarily involved the cortex, and usually only large lesions extended in to the medulla. The largest renal and splenic lesions were 15 and 20 mm in diameter respectively, and the largest lesion in an adrenal gland was a caseous lesion 17 mm in length.

Lesions observed incidentally, at sites other than the 40 routinely examined, included hypopyon of one eye, a 6 mm caseous nodule in the abdominal wall, and a caseous internal iliac and splenic lymph node. These possums were in an advanced stage of disease.

Microscopic lesions

The nature of microscopic lesions varied according to their size. The smallest lesions consisted of aggregations of a few large macrophages with angulated cytoplasmic boundaries (angulated macrophages), and lesser numbers of lymphocytes (Figure 3.10). These foci were easy to distinguish on low power when examining ZN stained sections, as they were paler than the surrounding tissue. Most affected tissues contained more than one tuberculous lesion, often of similar size, although it was not uncommon to see small satellite lesions surrounding larger ones. Randomly distributed neutrophils infiltrated large tuberculous lesions, which were often present in more expansive lesions in association with variable-sized foci of cells with pyknotic nuclei, and were usually accompanied by areas of coagulative or caseous necrosis (Figure 3.11). Liquefactive necrosis was not a feature of the lesions.

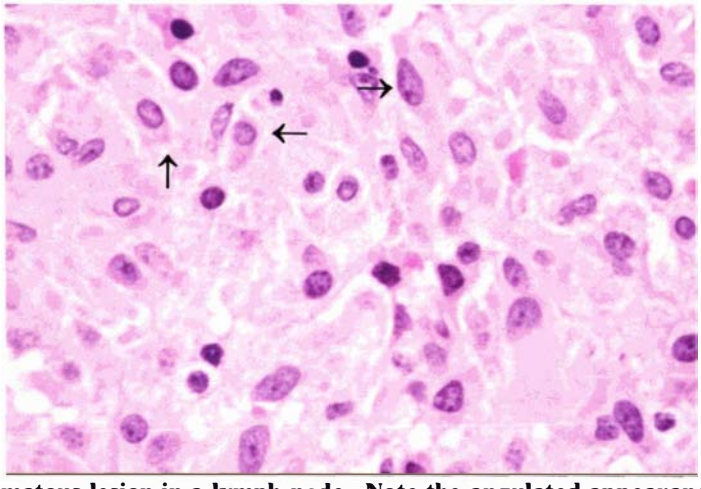

Figure 3.10 Granulomatous lesion in a lymph node. Note the angulated appearance of the macrophages (arrows). H&E. Magnification = 520x.

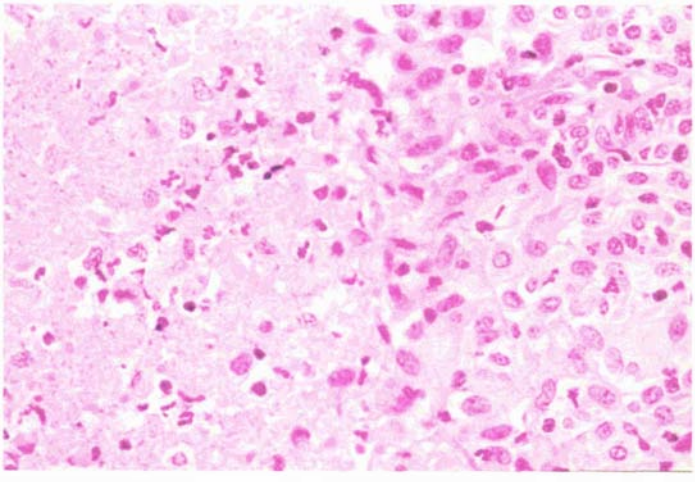

Figure 3.11 Caseation (left) merges into granulomatous inflammation (right). H&E. Magnification = 260x.

Even though AFOs were not always found in small granulomatous lesions, particularly those in the liver, a presumptive histological diagnosis of tuberculosis could often be made based on the typical morphological appearance of the aggregated macrophages. These differed morphologically from other clumps of plump macrophages containing phagocytosed particulate material in their cytoplasm that were also seen in lymph nodes and in the lungs of several non-tuberculous possums.

Acid fast organisms were mainly located within the cytoplasm of macrophages, although they were sometimes seen within neutrophils. Extracellular AFOs were common in necrotic foci, but were more numerous intracellularly at the periphery of these areas. The density of AFOs, assessed by taking the mean of 10 macrophages containing the highest numbers of AFOs from a total of 100 macrophages inspected, appeared to increase with increasing size of lesions (Figure 3.12). The maximum number of AFOs that could be counted in one section of a macrophage was 40. The density of AFOs within lesions in individual possums was relatively uniform, but was variable between individuals, and subjectively was not related to the number or extent of lesions.

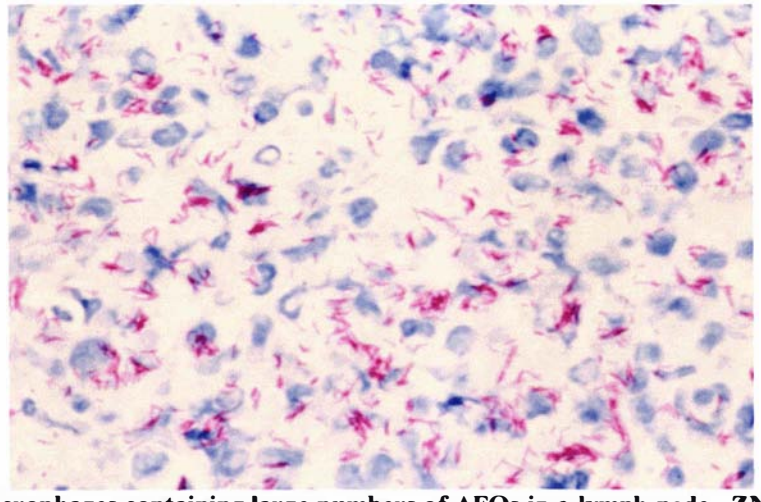

Figure 3.12 Macrophages containing large numbers of AFOs in a lymph node. ZN. Magnification = 560x.

Multinucleate giant cells were seen scattered randomly throughout a few lesions (Figure 3.13). Both Langhans'-type cells, with nuclei around the periphery, and foreign body-type giant cells, with the nuclei arranged throughout, were seen. Focal mineralisation of tuberculous lesions in lymph nodes was detected in three possums.

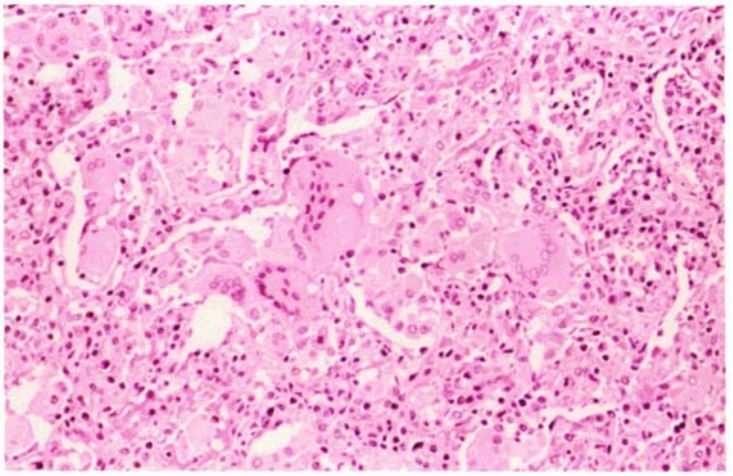

Figure 3.13 Multinucleated giant cells randomly distributed in a pyogranulomatous lesion in the lung. H&E. Magnification = 135x.

In most of the tissues examined, the margin of tuberculous lesions was poorly demarcated from the surrounding tissue. Fibroplasia was rarely observed, except when lesions occupied an entire lymph node, and the remaining lymph node capsule surrounding the lesion showed a mild fibroblastic reaction. Peripheral fibroplasia was occasionally seen in a few discharging superficial lymph nodes, as a discontinuous fibrous band around the periphery of the lesion. In the liver, and sometimes in the kidneys and adrenal glands, lesions were often more discrete, with compression of the surrounding tissue (Figure 3.14).

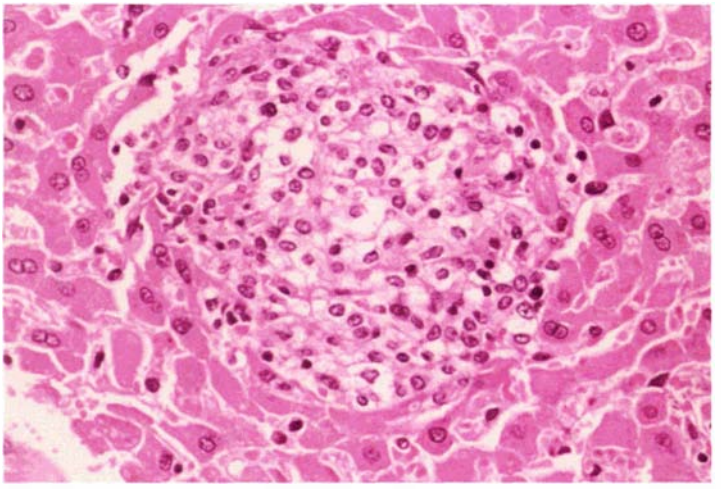

Figure 3.14 A small granulomatous focus in the hepatic parenchyma compressing adjacent tissue. H&E. Magnification = 265x.

Histological lesions were found in lymph nodes of all sizes (the smallest nodes in which lesions were seen being 1 mm in diameter). Multifocal lesions, often of varying size and extent, were common within the same node. Left deep axillary lymph nodes in which no macroscopic lesions were detected, were examined from 20 tuberculous possums. There was a variable

distribution of lesions within the entire node, with the majority (90%) of lesions located in the lower cortex or paracortex. Small granulomatous foci were occasionally seen in germinal centres, but were seldom seen in the medulla.

The smallest pulmonary lesions consisted of clumps of macrophages in alveolar spaces. Larger lesions involved more of the surrounding pulmonary parenchyma and often involved bronchioles. Many lesions were centred on blood vessels, causing distension of their walls and sometimes bulging into the vessel lumina. Macrophages and neutrophils containing AFOs were seen migrating between bronchiolar epithelial cells frequently in association with epithelial desquamation and necrosis. Similar mycobacterial-laden cells were often found in the bronchiolar lumina. In advanced lesions, large areas of the lung were consolidated, with few patent airways remaining. Involvement of pleural lymphatics was observed in one possum with extensive pleuropneumonia (Figure 3.15).

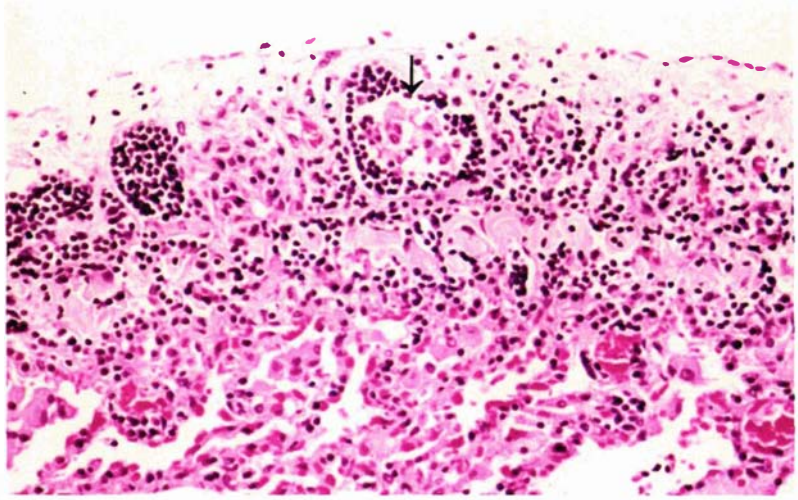

Figure 3.15 A small tuberculous focus inside a pleural lymphatic (arrow) of an affected lung. H&E. Magnification = 150x.

Two immature possums (H3126 and H3132) 6-8 months of age, and still living in close association with their dams, had a small number of lesions in their lower respiratory tract. In both possums, the lesions were centred on a bronchiole and alveolar spaces, with associated thickening of adjacent alveolar septa. In each case a granulomatous reaction was found protruding into the bronchiolar lumen. The largest lesion in one of these animals also involved a lymphatic adjacent to the affected bronchiole, and another lymphatic around a large vein. In another juvenile possum (R1895), in which the only macroscopic lesions were an 8 mm diameter caseous nodule in the left caudal lobe of the lung and enlargement of the left anterior mediastinal lymph node, microscopic lesions were detected only in these two sites. These comprised pyogranulomatous foci containing small numbers of AFOs. Unfortunately, the

morphology of the lung lesion in this case was complicated by the removal of tissue for mycobacterial culture. A further juvenile possum (G3553), which had a macroscopically caseous mesenteric lymph node measuring 12 x 17 mm, had an early microscopic lesion in the right cranial lung lobe, composed of a small necrotising granulomatous focus involving alveolar spaces.

Disseminated micro-granulomas were common in the hepatic parenchyma and a few of these also involved blood vessel walls (Figure 3.16). The smallest renal lesions were centred on the cortical interstitium and larger lesions obliterated adjacent cortical tubules and glomerular tufts. Renal blood vessel involvement was clearly evident in one possum. Acid fast organisms, although not numerous, were more commonly found in large pyogranulomatous renal lesions and were rarely seen in small aggregations of macrophages. Adrenal gland lesions were found throughout the medulla and cortex, but most were in the zonae fasciculata and reticularis of the cortex.

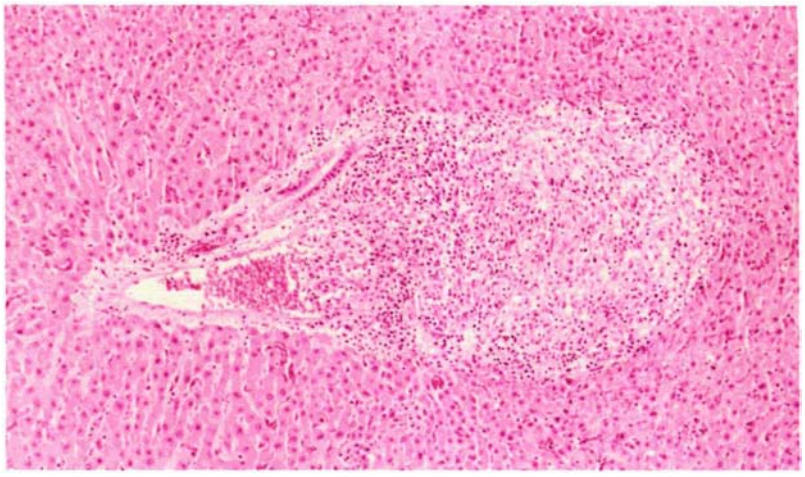

Figure 3.16 Involvement of an hepatic blood vessel in a granulomatous lesion in the liver. H&E. Magnification = 60x.

The smallest lesions in the spleen were situated around the periphery of periarteriolar lymphoid sheaths. Infiltration between the perivascular lymphoid cells occurred only in larger lesions. Very few AFOs were seen in splenic lesions. In the four (9.5%) female possums with microscopic lesions in mammary glands, the lesions were principally found around ducts of the teat sinus, and in the gland sinus of one possum. Some of the teat lesions extended into duct lumina and were associated with small numbers of AFOs.

Three (2.6%) possums had lesions in sections of the small and/or large intestines, which ranged from an aggregate of macrophages, containing several AFOs, to large pyogranulomatous foci, in

which numerous AFOs were seen. The lesions were located in the mucosa, Peyer's patches and intestinal wall. Acid fast organisms were also seen inside inflammatory cells amongst the contents of the intestinal lumen in one of these animals. There was no evidence that their presence in the intestinal lumen was an artefact of sectioning.

Other tuberculous lymphoid tissues observed included a lesion in the medulla of the thymus in a 2-year-old possum. In the palatine tonsils, lesions were seated within central areas of lymphoid nodules, but they sometimes also involved the lymphoepithelium. In these cases, a few AFOs were found between epithelial cells. In bone, small granulomatous lesions were seen randomly distributed in the marrow of the humerus (Figure 3.17). Some contained small numbers of AFOs, but these were not easily found because of loss of tinctorial quality of the section subsequent to the decalcification process. This problem was later overcome by scooping out the bone marrow for processing into paraffin blocks, thus omitting cortical bone, and the need for prior decalcification. Neutrophils were only seen in 25% of granulomatous lesions in bone marrow and there was no involvement of adjacent bone.

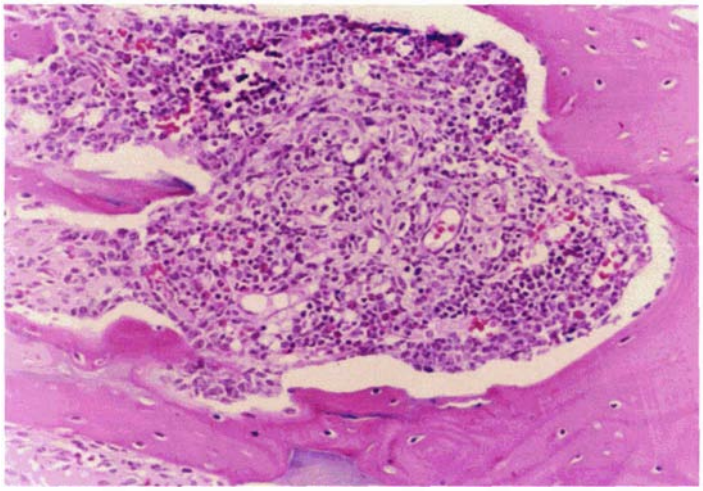

Figure 3.17 Granulomatous focus in the bone marrow of the left humerus. H&E. Magnification = 130x.

A dense mass of macrophages and neutrophils occupied the ciliary body and part of the iris of the left eye of the possum (D3694) with hypopyon, which also had extensive lesions in other tissues.

TERMINALLY ILL POSSUMS

Macroscopic lesions

There were 31 incidences of palpably or visibly enlarged, caseous, or discharging superficial lymph nodes, which occurred in 14 (70%) terminally ill possums, 10 (71%) of which were

males (Table 3.5). Eleven (79%) of these 14 possums had two or more macroscopically affected superficial nodes. Eleven (55%) terminally ill possums had discharging sinuses, eight (73%) of which were males. All possums with discharging sinuses had widespread, generalised disease.

Table 3.5 Nature and distribution of macroscopic lesions in superficial lymph nodes of 14 affected terminally ill possums

Lymph node[a]	Palpable/Enlarged	Caseous	Discharging	Total
L super axillary	1	3	3	7
R super axillary	0	1	2	3
L deep axillary	2	3	2	7
R deep axillary	1	3	1	5
L inguinal	0	2	1	3
R inguinal	0	2	4	6

[a]L = left; super = superficial; R = right

There was no difference between terminally ill and tuberculous possums in the nature of macroscopic lesions in lymph nodes. The largest lymph node lesion in this group was a caseous mesenteric node, which measured 70 x 20 mm. The normal range in size of this node, assessed in 56 non-tuberculous possums, was between 2-40 mm in length, and 1-11 mm in width.

Differences in the nature and distribution of lesions between the non-terminally ill and terminally ill possums were noteworthy (Table 3.6), and were most marked in the lung. However, all terminally ill possums had macroscopic lung lesions, and in all but two animals, all six lung lobes were affected. Macroscopic lung lesions were similar to those observed in non-terminally ill tuberculous possums, but were more widely disseminated and severe, with involvement of 50% of lung tissue or more. Pulmonary consolidation (Figure 3.18) and pleurisy were more common, affecting 12 (60%) possums. Two highly significant differences between the two groups of possums were the greater proportion of terminally ill possums with lesions in the intestines and mammary glands, indicative of shedding bacilli in the faeces and milk late in the course of the disease.

Macroscopic lesions in other tissues and organs had a similar appearance to those in tuberculous possums. The largest liver lesion was 8 mm in diameter, the largest renal lesion 9 mm in diameter, and the largest adrenal gland and splenic lesions 15 mm and 20 mm respectively. Mammary gland lesions were seen in one female, and involved a sinus discharging into the pouch. Extensive abdominal lesions were seen in the same animal, with cording of the

mesenteric lymphatics extending to involve the serosal lymphatics of the colon; the colonic wall was paler and thicker than normal.

Table 3.6 Differences in the nature and distribution of lesions between non-terminally ill and terminally ill possums

Feature	Non-terminally ill possums (n = 117)	Terminally ill possums (n = 20)	Statistical significance (χ^2, p value)
Mean no. of macroscopic lesions per possum	6.7	13.2	$\chi^2 = 2.9$, $p<0.1$
Mean no. of macroscopic + microscopic lesions	13.1	25.1	$\chi^2 = 7.2$, $p<0.01$
Largest lymph node lesion	60 x 50 mm	70 x 20 mm	Not applicable
% with discharging sinuses	30	55	$\chi^2 = 4.8$, $p<0.05$
% with macroscopic pulmonary TB	68	100	$\chi^2 = 8.7$, $p<0.01$
% of TB of the lung involving 6 lobes	45	90	$\chi^2 = 13.0$, $p<0.001$
% with pulmonary consolidation	24	60	$\chi^2 = 9.8$, $p<0.002$
% with pleurisy	9	60	$\chi^2 = 27.3$, $p<0.001$
% with (micro) granulomas in the liver	65	100	$\chi^2 = 9.9$, $p<0.002$
% with lesions in the intestines	2.6	63	$\chi^2 = 57.3$, $p<0.001$
% with mammary lesions	9.5	67	$\chi^2 = 12.3$, $p<0.001$

TB = tuberculosis

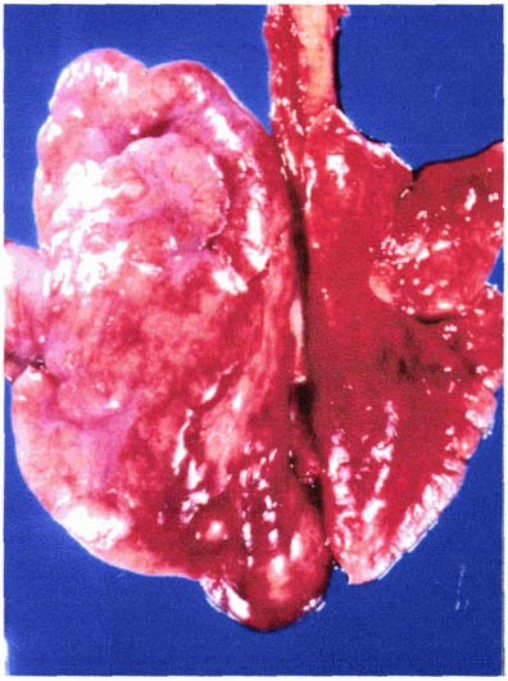

Figure 3.18 Lung from a terminally ill tuberculous possum. Most of the left lung is consolidated.

Tuberculous lesions observed incidentally at sites other than the 40 routinely examined included a 2 mm nodule in the diaphragm; lesions in two separate renal lymph nodes, one measuring 20 mm with liquid contents (Figure 3.19), and the other 30 mm but firm; a caseous internal iliac

lymph node of 28 mm; a 2 mm caseous nodule in the wall of the stomach; and a 2 mm caseous nodule in the myocardium.

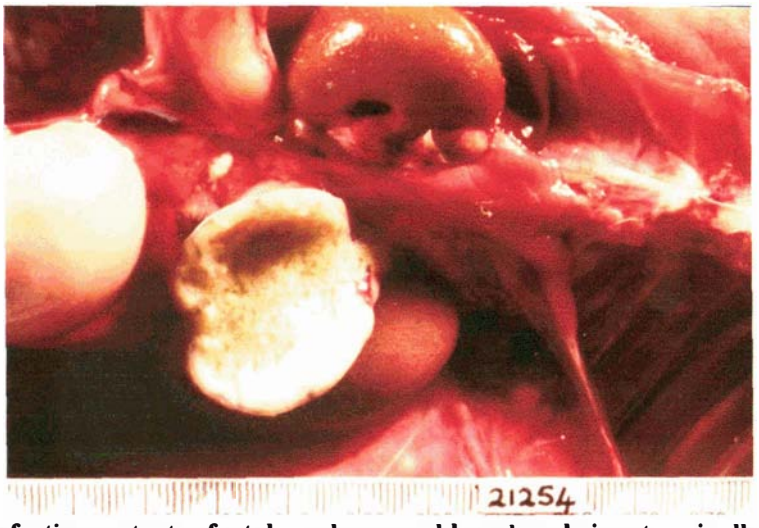

Figure 3.19. Liquefactive contents of a tuberculous renal lymph node in a terminally ill possum.

Microscopic lesions

The nature of microscopic lesions was similar to that observed in the non-terminally ill possums. A similar spectrum was observed in tissues in which no macrosocpic lesions were observed. However, at sites where macroscopic lesions were recorded, the histological lesions were commonly large, expansive, and necrotic and/or caseous. In all but two of the possums, AFOs were present in very high numbers, with most macrophages containing up to 40 AFOs in their cytoplasm. In the other two possums however, AFOs were present in low numbers despite the size and severity of the lesions.

Micro-granulomas were numerous throughout the hepatic parenchyma in all of the possums. Large numbers of AFOs were seen in a granulomatous focus in the lumen of a medullary tubule (Figures 3.20 and 3.21) near the renal pelvis in one possum.

The mammary glands of six of the eight females were available for histopathological examination. In four animals (67%), lesions occurred both around ducts of the teat sinus, as well as around the lactiferous glands in the gland sinus.

Of the 16 possums from which sections of the small and large intestine were sampled, 10 (63%) had lesions. Lesions were located in all three major layers of the intestine (mucosa, submucosa, muscle wall), as well as in the serosal lymphatics in three animals. Sloughing of cells containing AFOs into the intestinal lumen was frequently observed.

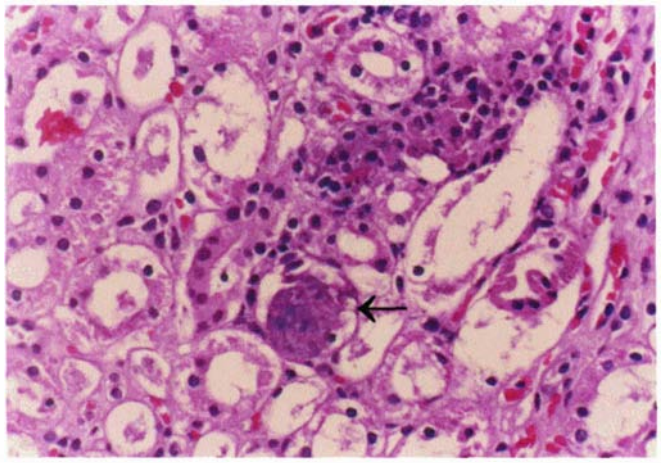

Figure 3.20 Micrograniuloma in a renal medullary tubule (arrow). Interstitial granulomatous inflammation is also evident above and to the right of the intra-tubular granuloma. H&E. Magnification = 240x. (ZN stained adjacent section illustrated in Figure 3.21).

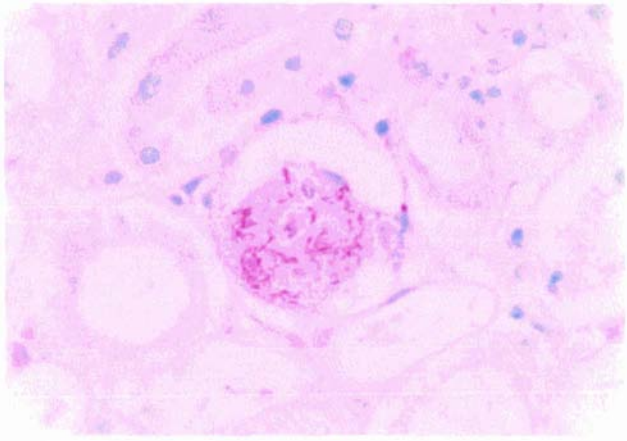

Figure 3.21 Potential urinary excretion of *Mycobacterium bovis* demonstrated by the presence of AFOs in an intra-tubular granuloma in the renal medulla. ZN. Magnification = 465x.

Additional tuberculous lesions detected histologically included two granulomatous foci containing AFOs beneath the squamous epithelium of the soft palate; several very small clusters of macrophages with moderate numbers of AFOs in the gastric pits of the fundic mucosa, and two small irregular foci of macrophages containing AFOs between and within individual muscle fibres in the myocardium of the left ventricle.

ULTRASTRUCTURAL STUDIES

Transmission electron microscopy on ultra-thin sections of lung and lymph node showed the presence of macrophages containing variable numbers of intracytoplasmic phagosomes (Figure 3.22), within which intact (presumably viable) tubercle bacilli were seen (Figure 3.23). Bacilli within phagolysosomes were not seen.

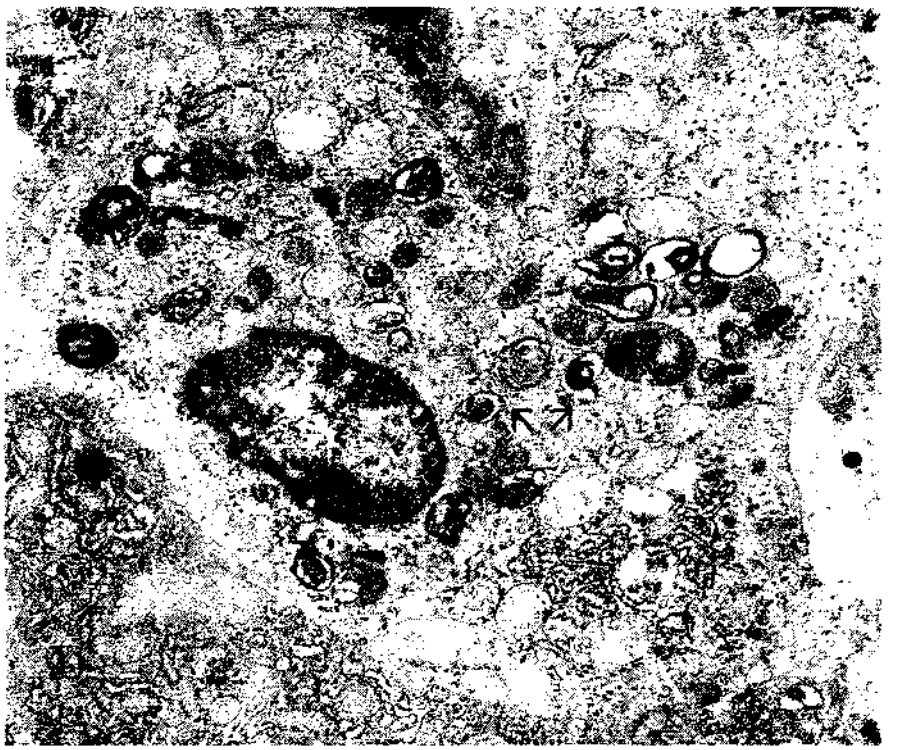

Figure 3.22 Macrophage from an infected lymph node. The cytoplasm contains a few tubercle bacilli inside phagosomes (arrows). TEM. Uranyl acetate-lead citrate. Magnification = 7800x.

Figure 3.23 High power view of *Mycobacterium bovis* inside a phagocytic vacuole. The phagosome wall is indicated by an arrow. An electron transparent zone (a) surrounds the bacillus. A central clear nnclear region (b) is evident. TEM. Uranyl acetate-lead citrate. Magnification = 103,600x.

3.4 DISCUSSION

This study used a large sample of animals for intensive pathological evaluation, and is the most comprehensive description of naturally occurring tuberculosis in possums undertaken to date. It has allowed inferences to be made about routes of infection and shedding, and formulation of hypotheses about the transmission and progression of *M. bovis* infection in possums. However, the absence of any practicable *in vivo* diagnostic test has made it difficult to determine the process and time frame of the disease as it relates to pathogenesis. Most of the data in this study were obtained through cross-sectional studies, which have the disadvantage of not giving an accurate picture of the temporal dynamics of the disease (Morris and Pfeiffer, 1991), but do not affect information with regard to the nature and distribution of lesions. Longitudinal studies on the other hand can detect any cyclic patterns in density or disease prevalence (Cheeseman *et al.*, 1989).

For the majority of the animals in this study, diagnosis of tuberculosis was confirmed by histopathology and the culture of *M. bovis* from macroscopic lesions, and in the remainder by histopathology alone, including the demonstration of the presence of typical lesions and associated AFOs. The collection of fresh specimens in the field can be complicated by cross-contamination and the consequent need for some use of a decontamination step, which reduces the chances of recovery of *M. bovis*. This would suggest that the overall disease prevalence diagnosed by cultural techniques would be conservative.

Although the gold standard for diagnosing tuberculosis is culture of the organism, in high prevalence areas, histopathological diagnosis may be satisfactory, but cannot be relied upon in all situations (Corner, 1994). In an earlier study at Hauhungaroa (Hickling *et al.*, 1991; Pfeiffer *et al.*, 1995), the diagnosis of tuberculosis was based solely on the presence of AFOs in histopathological lesions. In this study, histopathology was the sole means of diagnosis in 30 (20%) of the original pool of 151 tuberculous possums. *Mycobacterium bovis* has been the only mycobacterium isolated from macroscopic lesions in tuberculous possums (Julian, 1981). It was therefore considered acceptable to rely on histopathological diagnosis for tuberculosis in possums in some instances in this study, especially as *M. bovis* had been isolated from the population on previous occasions.

The sensitivity of histopathology for diagnosis was maximised by examination of a wide range of tissues from each animal, however the sensitivity for single tissues was limited by the need to confine examination to at most several 4 μm thick sections of tissue. Sensitivity for sites with

minimal microscopic lesions is poorer for large organs such as lung or liver than for small lymph nodes. In badgers, there is a high proportion of tuberculous animals which have no macroscopically visible lesions at post mortem examination (Gallagher *et al.*, 1976). To address this problem, Gallagher *et al.* (1998) made 1-2 mm thick sections of lung. When examined under an illuminated magnifying glass 20% (three of 15) had macroscopic lesions, and 87% had lesions when examined histologically. In this study, it was neither practicable nor desirable to slice the lungs very thinly. Instead, a consistent approach was taken when sampling the lung in those cases where no macroscopic lesions were evident. However, it is possible lesions could still have been missed.

As compared with the number of macroscopic lesions observed, the protocol used for histopathology resulted in a two-fold increase in the total number of detectable lesion sites within individuals, and identified a high prevalence of tuberculous lesions in lymph nodes of the head and neck. Thus previous studies of the naturally occurring disease, in which the diagnosis of tuberculosis was largely based on prominent macroscopic lesions, would undoubtedly have underestimated disease prevalence. In these studies, there were problems such as constraints on time, poor natural light, wet weather, and inter-operator variability. In cattle, the incidence of lung lesions is largely a reflection of how diligently the lungs are searched (McIlroy *et al.*, 1986), and the same is likely to hold true for possums, even though they have a much smaller lung mass and volume. The present study, though conducted in the field, followed a precise protocol, which allowed accurate detection and recording of lesions. A proportion of small lesions in some organs, particularly the liver and kidney, were found to be due to other causes, and histology was used to differentiate tuberculous from non-tuberculous lesions, thus increasing specificity of the diagnoses. These results support the belief of Hickling *et al.* (1991) that tuberculosis prevalence estimates in possums based on the identification of suspected cases from macroscopic lesions significantly underestimate the true prevalence. Of the possums which had no macroscopic lesions in the current study, eight of 46 (17%) were histologically positive for tuberculosis and a further 11 of 118 examined (9%) were culture-positive. Thus while studies based only on macroscopic lesions will generally underestimate the prevalence of tuberculosis in a population they may also overestimate the relative frequency of small lesions in lungs, liver and kidneys. This highlights the necessity for careful histopathological examination as an adjunct to all macroscopic examinations made when investigating the mode of infection of tuberculosis and its spread within the body (Innes, 1940).

The majority of possums in this study had multiple macroscopic lesions, with 31 of 99 (31%) possums in this study having single or multiple macroscopic lesions limited to a single body site. This number was further reduced to 17 (17%) when confined to a sole macroscopic lesion,

that is, a truly singular- lesion. This figure contrasts with that reported by Coleman (1988) who reported 55% and Pfeiffer *et al.* (1995) who found 57% of tuberculous possums with macroscopic lesions confined to a single body site. Lake (1975) had earlier reported eight of 26 (31%) tuberculous possums with a single macroscopic lesion. These differences are attributed to the use of a more detailed necropsy procedure in the current study.

Single site lesions are considered indicative of the route of infection (Francis, 1958). The lesion in 14 of the 17 (82%) animals in this study with single macroscopic lesions was seated in the superficial lymph nodes, seven (50%) of which were in the left superficial axillary lymph node. Of the remaining three cases of single macroscopic lesions, two were in the lung, and one was in the mesenteric lymph node. Assessment at the microscopic level resulted in only five (4.3%) possums having a single lesion – mesenteric lymph node (one animal), inguinal lymph node (two animals), superficial axillary lymph node (two animals). Because too few animals had single lesions for any statistically significant conclusions to be made, it appears that the concept of the primary complex cannot be easily applied to possums. However, the lesion distribution suggests that the superficial lymph nodes are a predilection site for the deposition of *M. bovis* and for the development of lesions.

The full spectrum of the disease was demonstrated in this study, ranging from NVL animals with microscopic lesions, through macroscopic lesions at multiple sites, to the terminally ill state. But, it is not possible to determine the sequence of lesion development. An important finding in this study has been the potential for simultaneous spread of the disease by blood vessels, airways and lymphatics which was noted in tissues such as the lung, liver, kidney, lymph nodes and colon. However, it is not possible to determine whether the direction of this spread was towards or away from these tissues. Although there was histological evidence consistent with both haematogenous and lymphatic spread in lymph nodes, liver and lungs, it could not be established in which order these occur, but it appears that lymphatic and haematogenous spread occur early in the course of the disease. This is supported by the fact that only five possums had a single macroscopic and/or microscopic lesion, indicating rapid extension to other sites.

Lesions in spleen, bone marrow, kidneys, adrenal and mammary glands are considered indicative of generalised disease in other mammalian species (Francis, 1958) and almost certainly result from haematogenous spread of organisms. Macroscopic and microscopic lesions were found in these sites in 58% of tuberculous possums, suggesting haematogenous spread is common and occurs relatively early in the disease process. Seventy-three percent of tuberculous possums had lesions in the liver, and this infection may also have come about by

haematogenous spread. The liver is associated with the GIT, through the excretion of bile, and because all the material absorbed via the intestines reaches the liver through the portal vein, or, in the case of lipids, mainly by lymph vessels. Although it is not possible to separate out the contribution of hepatic granulomas attributable to systemic haematogenous spread from those arriving via the portal system, 61% of tuberculous possums had lesions in their mesenteric lymph nodes.

Thirty-five of the 88 (40%) tuberculous possums with macroscopic lesions in superficial lymph nodes had lesions discharging from at least one node through a sinus to the skin, and was correlated with infection of all six lobes of the lung. Similarly, Hickling *et al.* (1991) noted discharging sinuses in 38% of tuberculous possums in their study. Open suppurating sinuses draining infected lymph nodes have been found more commonly in animals with advanced disease (Stockdale, 1975; Anonymous, 1977; Hickling *et al.*, 1991), and this has been confirmed by findings in the terminally ill group of possums in this study. While the majority of the tuberculous possums with discharging sinuses had generalised disease, four had very few macroscopic lesions. One animal (H3181) had only two individual macroscopic lesions, and in the other three possums the discharging sinus was the only macroscopic lesion observed. Most affected animals are likely to be excreting organisms by at least one route other than the discharging sinus.

The pathology of naturally occurring tuberculosis in possums has recognisable differences from that reported in badgers and ferrets. Gallagher *et al.* (1998) described early granulomas in tuberculous badgers as round, cellular nodules of mainly epithelioid cells, with a small, central necrotic focus of cellular debris and pyknotic nuclei. A few neutrophils were often present in the necrotic focus. Peripherally, lymphocytes and macrophages were encased in a mild fibroblastic reaction. Small numbers of AFOs were sometimes present, mostly in the centre, but were more often absent. More advanced lesions had more pronounced central necrosis and slightly greater fibroblastic activity. The numbers of AFOs increased with the severity of the cellular reaction. The presence of well-defined coagulative necrosis was almost invariably associated with numerous AFOs. Variable mineralisation of small, sharply defined, fibrous encapsulated pulmonary lesions with central coagulative necrosis was observed. Giant cells were not seen. Lugton *et al.* (1997b) described the inflammatory reaction in tuberculous ferrets as comprised of discrete aggregates of epithelioid cells. There was often a central core of necrotic tissue, and the reaction was surrounded by a thin margin of lymphocytes and lesser numbers of plasma cells. Acid fast organisms, which were present in low numbers, were rarely present in necrotic areas, but more commonly in epithelioid cells around the margin of necrosis.

Increasing necrosis was reflected in increasing numbers of AFOs. Fibrosis was not prominent, neutrophils were infrequent, and there was no record of mineralisation or giant cells.

Compared with these two species of mustelids, lesions in tuberculous possums are less discrete. They are characterised by the presence of angulated macrophages, not epithelioid cells, and are predominantly pyogranulomatous in nature. Giant cells characteristic of the disease in many species were also rarely seen as in previous studies (Cook and Coleman, 1975; O'Hara *et al.*, 1976; Corner and Presidente, 1980). Both Langhans' and foreign body-type giant cells were recorded in the current study. Although emphasis has been placed on the importance of Langhans' giant cells in tuberculous lesions, Thomson (1978) believed that the distinction between the two types of giant cells was not valid. He stated that was because both types were found in the same lesion and there seemed to be no relationship between the type and an aetiological agent. Although possums do not form classical granulomas with *M. bovis* infection, nevertheless they do have the ability to form granulomas in response to foreign bodies and parasites (pers. obs.). In tuberculous lesions in possums, giant cells were admixed into the granulomatous inflammatory reaction. In contrast, in the classical tubercle of *M. bovis* infection in cattle, giant cells are arranged around the periphery of necrotic foci. Therefore, it may be confusing to use the term "tubercle" when referring to lesions in tuberculous possums.

The increasing number and size of histological lesions and the associated increasing density of AFOs probably represent the natural progression of the disease although no information on their rate of development could be obtained. However, very few AFOs were seen in granulomatous lesions in the liver, a finding previously reported by Corner and Presidente (1980). A similar histological association between lesion severity and numbers of AFOs was also noted in tuberculous badgers (Gallagher *et al.*, 1998) and ferrets (Lugton *et al.*, 1997b). In their experimental studies of *M. bovis* infection in possums, O'Hara *et al.* (1976) observed that the density of AFOs increased with increasing size and caseation of lesions, but that their number decreased once liquefaction occurred. This conflicts with the findings in terminally ill possums in the current study, where AFOs were extremely numerous. It also contrasts with Bates (1980) who states that tubercle bacilli are able to grow rapidly after liquefaction occurs in areas of caseation. From personal observation of liquefactive lesions in tuberculous lymph nodes from ferrets, it can be difficult to visualise AFOs due to a partial loss of their tinctorial quality, as loss of integrity of the cell wall results in loss of acid fastness.

In experimental studies of tuberculosis in possums, Buddle *et al.* (1994) described small lesions consisting of aggregations of macrophages and a few neutrophils, and larger lesions with extensive necrosis and numerous AFOs. Although Buddle *et al.* (1994) and Pfeffer *et al.* (1994)

noted the presence of neutrophils in small lesions these were not seen in the smallest lesions in this study. However, O'Hara *et al.* (1976) described the earliest experimental lesion as focal necrosis infiltrated by neutrophils before invasion by macrophages and, as lesions enlarged, so too did the extent of coagulative necrosis until caseation resulted.

Fibrosis in any tissue was rare, as noted in other investigations (Lake, 1975; Corner and Presidente, 1980). Mineralisation of tuberculous lesions has not previously been recorded (Lake, 1975; Julian, 1981), but was reported for the first time in a very small number of possums in this study. Dystrophic calcification is commonly observed in tuberculosis in cattle, and has been recorded in badgers (Gallagher *et al.*, 1998). Tuberculous lesions in both these species are more discrete than in possums, and the disease is of a longer duration. The scarcity of mineralisation of lesions in possums may be related to the expansive nature of lesions, and may also be a reflection of the rapidity of the disease. Bates (1980) provided support for this theory when he wrote that if bacillary replication is contained in areas of caseous necrosis, calcium may begin to be deposited in their centres after about 3 months.

Ultrastructural studies of mycobacterial species have largely been confined to studies in experimentally infected animals, pellets of tubercle bacilli, or macrophages infected with tubercle bacilli (D'Arcy Hart *et al.*, 1972; McDonough *et al.*, 1993), and have seldom involved *M. bovis* (Rastogi *et al.*, 1986). Although this is the first known documentation of the ultrastructure of *M. bovis* in any naturally infected mammalian species, it confirms experimental findings of the presence of a virulent mycobacterial species inside phagosomes in the cytoplasm of macrophages (Armstrong and D'Arcy Hart, 1971; McDonough *et al.*, 1993). McDonough *et al.* (1993) provided electron microscopic evidence that tubercle bacilli escape from a fused phagolysosome into another membrane-bound vesicle, which results in resistance to fusion with secondary lysosomes, and thus protection of the bacillus. Phagolysosome fusion was observed in infected macrophages by these authors as early as 2 hours p.i., but had completely reversed after 3 to 4 days. As the ultrastructural findings from the current study were from macroscopically visible lesions, this early phenomenon of escape from fused phagolysosomes is unlikely to have been observed.

In this study, lymph node lesions were found throughout the node, predominantly in the lower cortex and paracortex, but the early splenic lesions were peri-arteriolar. This contrasts with the experimental observation of Corner and Presidente (1980) that in both these tissues, lesions were centred on lymphoid follicles. However, the histological studies in the experimental work of Pfeffer *et al.* (1994) found that splenic lesions were located around the periphery of periarteriolar lymphoid sheaths and lymph node lesions were in the paracortex. In eutherians,

tuberculous lesions in the outer cortex of lymph nodes are considered likely to have arisen from lymphatic infection of the node, whereas those in the paracortex are more likely to have been blood-borne (Junqueira and Carneiro, 1980). Lesions were observed in both of these locations in this study, but were more common in paracortical areas. It should be noted that at least some marsupials do not have the same lymphatic flow patterns within nodes as eutherians (Hopwood, 1980), so lesion site in the node may not be an indication of the route of infection.

The location of adrenal gland lesions in the lower cortex and medulla is likely to be due to the rich vascularisation of this region of the gland (Junqueira and Carneiro, 1980). The finding of a tuberculous lesion in the thymus of one possum is considered rare, although thymic involvement has also been reported in two badgers (Gallagher *et al.*, 1976; Clifton-Hadley *et al.*, 1993).

Most possums in this study were sampled during August or December. Due to the breeding season of the possum, a greater proportion of immature possums was available in December than in August. However, exclusion of immature possums captured in December resulted in possums similar in age to those in August samples (Coleman and Cooke, 2000). It is therefore likely that the sample of non-terminally ill possums includes animals at all stages of the disease. However, it cannot be said with any certainty that no terminally ill possums were included in the group of 117 tuberculous possums sampled. This is because the traps were checked only once every 24 hours, and although the great majority of possums would have been caught overnight it was possible, but not likely that a terminally ill possum was trapped while wandering in daylight hours.

The disease in terminally ill possums had more features in common with that described in experimental infections, in which high doses of *M. bovis* were administered (Corner and Presidente, 1980, 1981), than early cases of the natural disease. Terminally ill possums had twice as many macroscopic lesions as the non-terminally ill tuberculous possums, and lesions were generally larger, and more expansive. Lung lesions were frequently those of consolidation. It is speculated that the aberrant behaviour exhibited by these animals in the last few days of their lives is due to a combination of effects. This could include the effects of toxic by-products of the extensive inflammatory and necrotising lesions. The animals could also be weak and hypoglycaemic due to the reduction or cessation of dietary intake. Death would ensue as a result of these effects, combined with pulmonary and/or multiple organ failure. Histological evidence of excretion via urine, and faeces in particular, was demonstrated in the majority of terminally ill possums, but was a rare event in the non-terminally ill possums. In experimental infections, excretion commonly occurred in the urine and faeces (Corner and

Presidente, 1980, 1981), and animals succumbed to the infection in a matter of 4-5 weeks. The present study indicates that in possums naturally infected, urinary and faecal shedding of *M. bovis* occur very late in the course of the disease.

Shedding of mycobacteria from the mammary gland is a well recognised phenomenon in cattle and goats (Stamp, 1944; Soliman *et al.*, 1953) and usually occurs late in the course of the disease. Tuberculous lesions were also found in mammary glands of possums with advanced lesions in this study, particularly in terminally ill animals. As young possums spend the first few months of life attached to the teat, and are regularly suckled to weaning at approximately 6 months, excretion by this route could be very important in transmission from mother to pouch young. Lesions were found in dependent juveniles in this study, confirming the occurrence of pseudo-vertical transmission. This may occur either from the mammary gland or from the oropharynx or respiratory tract through grooming and other attentions of the mother to her young.

The prevalence of tuberculosis was greater in males than females, despite compensating for a greater proportion of males in the trapped population from which the tuberculous possums were sourced. Also, proportionately more males than females had macroscopic lesions in superficial lymph nodes and lung, as well as discharging sinuses. Lesions have been more commonly observed in male possums previously (Coleman, 1988). This has been attributed to behavioural differences between the two sexes, namely the greater mobility and enhanced trappability of males (Pfeiffer *et al.*, 1995), as well as a greater frequency of antagonistic encounters between males, especially during the mating season (Julian, 1981; Morris and Pfeiffer, 1995). More infected males than females were found on the forest-pasture margin (Coleman, 1988). Hickling *et al.* (1991) found that disseminated lesions were more common in immature males than immature females, and that lesions in the GIT were more common in adult females than adult males. Possible reasons for the latter phenomenon included allogrooming, self-grooming, marking behaviour, or consumption of contaminated pasture. Males had a mean of 1.9 lesions each compared with 1.7 lesions each in females (Cook and Coleman, 1975).

The pathological findings reported here have confirmed that tuberculosis in the possum is a disseminated disease, often producing small histological lesions in multiple tissues and organs. The findings suggest that the disease initially develops rapidly, but progression to clinical disease is variable until death occurs due to either respiratory or multiple organ failure.
Most importantly, they raise questions concerning the predominance of infection in superficial lymph nodes and the absence of macroscopically visible lesions in the skin which drains to these lymphocentres.

CHAPTER 4. EXPERIMENTAL RESPIRATORY INFECTION WITH *MYCOBACTERIUM BOVIS*

4.1 INTRODUCTION

Earlier studies of naturally occurring tuberculosis in wild possums have revealed that the lungs are the most common site for macroscopic lesions (Julian, 1981; Coleman, 1988) and it was therefore concluded that the most likely route of infection was the respiratory tract (Jackson *et al.*, 1995b). Although many of these studies were detailed, they were unable to explain the high frequency of lesions outside the respiratory tract (Chapter 3), or provide information on the routes of spread, progression and duration of respiratory infection before the possums became terminally ill.

Previous experimental approaches have involved infecting possums with low doses of *M. bovis* via the endo-bronchial (E/B) and intra-tracheal (I/T) routes, in an attempt to model the natural disease (Buddle *et al.*, 1994; Pfeffer *et al.*, 1994). Extensive pulmonary changes were observed by these authors when animals were killed 6-8 weeks after inoculation, with lesions ranging from miliary through to nodular and consolidated lung lobes. Nodular tuberculous lesions were also common in the liver, spleen and kidneys. Despite the range of lesions produced, the data derived from these experimental studies provided little information on the progression of lesions in the natural disease. The experiments nevertheless showed that possums are very susceptible to low doses of *M. bovis* via the pulmonary route, and progression of the disease is dose-dependent. Lesions which occurred in mandibular, deep and superficial cervical, and axillary lymph nodes were thought to be due to lymphatic drainage of injection site lesions in the ventral neck. Lesions elsewhere were indicative of lymphatic and haematogenous spread from pulmonary lesions. The low dose rate produced a result which best fitted the nature and extent of lesions seen in natural infections.

The studies presented here were designed to follow early events and the sequential appearance of lesions following the infection of possums by means of E/B and aerosol methods with a low dose of viable *M. bovis*. They aimed to compare experimental respiratory infection with the natural disease, and study how experimental respiratory lesions develop in relation to lesions in other tissues, particularly those in peripheral lymph nodes.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Fifty-three adult possums (37 males, 16 females), weighing 2-3.5 kg, were captured from the Hutt Valley region of New Zealand. The possums were housed in individual wire cages with externally fitted nesting-boxes (Pfeffer *et al.*, 1994), and were fed a diet of carrots, apples, pasture weeds and commercial possum pellets (Sharpes Grain and Seeds Limited, Lower Hutt), with water available *ad libitum*. They were housed for a minimum of 3 weeks prior to the experiments to decrease the effect of stress associated with capture and housing in cages (Buddle *et al.*, 1992). To facilitate handling, the possums were anaesthetised with an intramuscular injection of ketamine HCl (Pfeffer *et al.*, 1994).

4.2.2 Experimental design

The possums were inoculated with *M. bovis* strain 83/6235, which was originally isolated from the mandibular lymph node of a wild possum from Taumarunui, New Zealand (Buddle *et al.*, 1994), and grown to mid log phase in Tween albumin broth (Buddle *et al.*, 1994).

ENDO-BRONCHIAL INOCULATION

Anaesthetised possums were inoculated with 0.2 mL of inoculum containing about 20-100 colony forming units (cfu) of *M. bovis*, via a 2 mm diameter cannula inserted through the mouth deep into the trachea (Pfeffer *et al.*, 1994). The inoculum was flushed through the cannula with 0.1 mL of sterile saline. Following inoculation, the possums were placed on their right side to recover. Seven possums were humanely killed by CO_2 inhalation at 1 week post inoculation (p.i.), eight possums each at 2 and 3 weeks p.i., and 10 possums at 4 weeks p.i. (Table 4.1).

Table 4.1 Weeks post inoculation at which 53 possums inoculated either via the endo-bronchial route or by aerosol were humanely killed

	No. of animals killed post inoculation (p.i.)					
Means of inoculation	1 week p.i.	2 weeks p.i.	3 weeks p.i.	4 weeks p.i.	5 weeks p.i.	Total
Endo-bronchial	7	8	8	10	N/A	33
Aerosol	4	4	4	4	4	20

N/A = not applicable.

AEROSOL INOCULATION

Single cell suspensions of the *M. bovis* isolate were prepared using a modification of the method described by Grover *et al.* (1967) and stored at –70°C. For preparing these suspensions, the bacterial cells were dispersed by sonication for 30 seconds and filtered through an 8 μm membrane filter. Anaesthetised possums were infected via the respiratory route by using an aerosol-generating chamber which produces droplet nuclei of the size appropriate for entry into alveolar spaces (Wiegeshaus *et al.*, 1970; McMurray *et al.*, 1985). The anaesthetised possums were put into individual tubular wire-mesh baskets (180 mm diameter x 450 mm long), and three possums were placed in the aerosol chamber for each aerosol run. The possums were exposed to an aerosol of *M. bovis* for 5 min., followed by 10 min. of air alone. The concentration of viable *M. bovis* in the nebuliser fluid was empirically adjusted to result in the inhalation and retention of 10-20 viable organisms per possum (Buddle and de Lisle, unpublished). This challenge dose had previously been estimated from the number of primary tubercles observed macroscopically in the lungs of susceptible possums at 4 weeks p.i. A similar procedure has been shown to result in reproducible, uniform infection of the lungs of guinea pigs (Smith *et al.*, 1970a; Wiegeshaus *et al.*, 1970). The aerosol infection and subsequent maintenance and manipulation of infected possums were performed under strict isolation conditions in a biohazard facility at AgResearch, Wallaceville. Four possums were humanely killed by CO_2 inhalation at each of 1, 2, 3, 4 and 5 weeks p.i. (Table 4.1).

4.2.3 Necropsy

The animals were necropsied and tissues were collected for histopathological examination from the 40 separate body sites listed by Cooke *et al.* (1995). After fixation in 10% neutral buffered formalin, all tissues were incised, and the size, nature and distribution of macroscopic lesions were recorded. Paraffin embedded tissues were cut at 4 μm, and stained with haematoxylin and eosin (H&E) and Ziehl-Neelsen[1] (ZN) stain.

Assessment of the numbers or density of acid fast organisms (AFOs) was performed on the largest lesion in each lung of each possum, which involved areas with the greatest density of AFOs. For possums infected via the E/B route, intra- and extra- cellular AFOs were counted in ten 0.017 x 0.017 mm squares using an eyepiece graticule at high power (600x magnification). For possums inoculated via aerosol, the density of AFOs was scored by assessing the mean of

[1]Culling CFA, Allison RT, Barr WT (eds). Cellular Pathology Technique. 4th Edtn. P 336. Butterworths, London, 1985.

10 macrophages containing the highest numbers of AFOs, from a total of 100 macrophages examined.

The anterior mediastinal lymph nodes were selected for a detailed histological study of lesion development over time, in association with the density of AFOs scored as described above. These nodes were fixed whole and sectioned longitudinally. Lesion types were divided into four histological stages of development, based on their age, size and histological nature, and the dominant lesion stage recorded each week p.i.

Following necropsy of the possums in the E/B experiments, the lungs were lavaged with warm sterile phosphate buffered saline (PBS), pH 7.2, massaged, and the cells and fluid expressed by gentle compression. Cell suspensions thus harvested were centrifuged at 350 **g** at room temperature for 10 min. The pellet containing alveolar macrophages was utilised for ultrastructural studies. For electron microscopy, the alveolar macrophages were fixed in 2% gluteraldehyde overnight, then post-fixed in 1% osmium tetroxide in PBS, pH 7.4, for 1 h and embedded in epoxy resin (Procure 812, Probing and Structure, Thuringowa, Queensland, Australia). Thin sections were cut and mounted on copper grids before staining with uranyl acetate and lead citrate, and the grids examined by transmission electron microscopy (TEM) (Philips 201c TEM).

4.2.4 Bacteriology

The distal half of the right accessory lung lobe of the possums infected via the E/B route was collected aseptically and mycobacterial culture was undertaken using the methods described by Buddle *et al.* (1994). For possums infected via aerosol, the entire right accessory lung lobe was taken for mycobacterial culture, and was therefore unavailable for histopathological examination.

4.2.5 Statistical analysis

Spearman's rank correlation coefficient was used to assess whether bacterial counts and AFOs seemed to be related, either across weeks, or within a group of animals in a particular week. The statistical significance of these was tested by applying the least significant difference (LSD) test, and conducted with the aid of a statistical software package (Statistix, Analytical Software Co., La Jolla, California).

4.3 RESULTS

As no clinical signs of disease were observed in any of the inoculated animals, all possums were humanely killed as scheduled.

4.3.1 Macroscopic lesions

ENDO-BRONCHIAL INFECTION

Macroscopic lesions were first detected at 2 weeks p.i. (Table 4.2), and were confined to the right lobe(s) of the lung. The lung lesions were greater in both size and number in the possums killed 3 and 4 weeks p.i. (Table 4.3). They were nodular creamy-white masses ranging from 1 mm in diameter at week 2, to 25 mm in diameter at week 4. By the third week p.i., lesions were also seen in the left caudal lung lobe of one possum. At this time, the anterior mediastinal (bronchial) lymph nodes were visibly enlarged (up to 15 mm in length) in seven possums and areas of caseation were observed in the remaining possum. By week 4, areas of consolidation were observed in the lungs of seven possums, often involving an entire lung lobe. At this stage, all lung lobes were affected, and all the possums had caseous lesions in the anterior mediastinal lymph nodes. The only macroscopic lesions detected in any of the other body sites outside the lower respiratory tract were a small number of randomly distributed cream nodules 1 to 2 mm in diameter in the liver of two possums at week 4.

Table 4.2 Distribution of macroscopic lesions in possums killed at weekly intervals following endo-bronchial inoculation with about 20-100 cfu of viable *Mycobacterium bovis*

	Number (%) of possums with macroscopic lesions			
Lesion site	Week 1 (n = 7)	Week 2 (n = 8)	Week 3 (n = 8)	Week 4 (n = 10)
Left cranial lung lobe	0	0	0	2 (20%)
Left caudal lung lobe	0	0	1 (13%)	2 (20%)
Right cranial lung lobe	0	0	0	2 (20%)
Right middle lung lobe	0	0	4 (50%)	8 (80%)
Right caudal lung lobe	0	2 (25%)	6 (75%)	7 (70%)
Right accessory lung lobe	0	1 (13%)	2 (25%)	5 (50%)
Left anterior mediastinal lymph node		0	6 (75%)	7 (70%)
Right anterior mediastinal lymph node	0	0	5 (63%)	10 (100%)
Liver	0	0	0	2 (20%)

cfu = colony forming units

Table 4.3 Average number of nodules in the lung of each possum following endo-bronchial and aerosol inoculation with *Mycobacterium bovis*

	Mean (and range) of total number of macroscopic nodules per animal in the lung[a]			
Route of inoculation	Week 2	Week 3	Week 4	Week 5
Endo-bronchial	2 (range = 2-2)	4 (range = 1-7)	6 (range = 1-18)	Not applicable
Aerosol	0	5 (range = 2-9)	10 (range = 5-14)	15 (range = 10-29)

[a]Excludes the right accessory lung lobe, which was not consistently available for assessment

AEROSOL INFECTION

Macroscopic lesions were first detected at 3 weeks p.i. (Table 4.4), and were confined to the lower respiratory tract. The lung lesions were greater in both size and number in the possums killed 4 and 5 weeks p.i. (Table 4.3). They were nodular creamy-white masses ranging from 1 to 3 mm in diameter at week 3, through 3-11 mm in diameter at week 4, to 30 mm in diameter at week 5. By the fifth week p.i., pleural adhesions were observed on the affected lungs of three animals. One possum each at weeks 4 and 5 p.i. had all lung lobes affected. At 3 weeks p.i., the left anterior mediastinal (bronchial) lymph node of one possum was visibly enlarged (up to 12 mm in length), and both left and right anterior mediastinal lymph nodes were macroscopically affected in all but one possum at week 4 p.i. At week 5 p.i., both left and right anterior mediastinal lymph nodes were caseous in all possums. The only macroscopic lesions detected in any of the other body sites outside the lower respiratory tract occurred in one possum at week 4 p.i. They were numerous randomly distributed cream nodules 1 to 2 mm in diameter in the liver, a 4 mm caseous nodule in the swollen spleen, and visibly enlarged gastric, hepatic and deep cervical (retropharyngeal) lymph nodes.

Table 4.4 Distribution of macroscopic lesions in possums killed at weekly intervals following aerosol inoculation with about 10-20 viable *Mycobacterium bovis*

	Number (%) of possums with macroscopic lesions				
Lesion site	Week 1 (n = 4)	Week 2 (n = 4)	Week 3 (n = 4)	Week 4 (n = 4)	Week 5 (n = 4)
Left cranial lung lobe	0	0	2 (50%)	4 (100%)	2 (50%)
Left caudal lung lobe	0	0	2 (50%)	4 (100%)	4 (100%)
Right cranial lung lobe	0	0	1 (25%)	1 (25%)	2 (50%)
Right middle lung lobe	0	0	2 (50%)	4 (100%)	2 (50%)
Right caudal lung lobe	0	0	1 (25%)	4 (100%)	4 (100%)
Right accessory lung lobe	0	0	0	2 (50%)	3 (100%)
Left anterior mediastinal lymph node	0	0	1 (25%)	3 (75%)	4 (100%)
Right anterior mediastinal lymph node	0	0	0	4 (100%)	4 (100%)
Liver; spleen; gastric, hepatic and deep cervical lymph nodes	0	0	0	1 (25%)	0

4.3.2 Histological lesions

Histopathological examination greatly increased the detection of lesions in both groups, especially in tissues other than the lung (Tables 4.5 and 4.6).

Table 4.5 Distribution of microscopic lesions in possums killed at weekly intervals following endo-bronchial inoculation with about 20-100 cfu of viable *Mycobacterium bovis*

	Number (%) of possums with microscopic lesions			
Lesion site	Week 1 (n = 7)	Week 2[a] (n = 8)	Week 3 (n = 8)	Week 4[a] (n = 10)
Right superficial axillary lymph node	0	0	0	1 (10%)
Left deep axillary lymph node	0	0	0	6 (60%)
Right deep axillary lymph node	0	0	0	2 (20%)
Left inguinal lymph node	0	0	0	2 (20%)
Right inguinal lymph node	0	0	0	4 (40%)
Left mandibular lymph node	0	0	0	1 (10%)
Right mandibular lymph node	0	0	0	1 (10%)
Left parotid lymph node	0	0/6	0/6	1/9 (11%)
Right parotid lymph node	0	0/7	0	1/8 (13%)
Right deep cervical lymph node	0	0	0	1 (10%)
Right superficial lymph node	0	0	0	1 (10%)
Left cranial lung lobe	0	0	1 (13%)	5 (50%)
Left caudal lung lobe	0	1 (13%)	2 (25%)	7 (70%)
Right cranial lung lobe	0	0	0	7 (70%)
Right middle lung lobe	0	2 (25%)	7 (88%)	9 (90%)
Right caudal lung lobe	0	5 (63%)	7 (88%)	10 (100%)
Right accessory lung lobe	0	2 (25%)	3 (38%)	7 (70%)
Left anterior mediastinal lymph node	0	0/6	6 (75%)	8 (80%)
Right anterior mediastinal lymph node	0	2/4 (50%)	8 (100%)	10 (100%)
Mesenteric lymph node	0	0	0	5 (50%)
Gastric lymph node	0	0	0	2 (20%)
Hepatic lymph node	0	0	1 (13%)	9 (90%)
Liver	0	0	1 (13%)	7 (70%)
Spleen	0	0	0	6 (60%)
Bone marrow	0	0	0	2 (20%)
Left kidney	0	0	0	2 (20%)
Right kidney	0	0	0	1 (10%)
Left adrenal gland	0	0	0	2 (20%)
Right adrenal gland	0	0	0	1/9 (11%)
Right mammary gland	0	0	0	1/5 (20%)

cfu = colony forming units

[a]The denominator refers to the number of observations where samples were not examined from all animals at that time.

Table 4.6 Distribution of microscopic lesions in possums killed at weekly intervals following aerosol inoculation with about 10-20 viable *Mycobacterium bovis*

Lesion site	Number (%) of possums with microscopic lesions				
	Week 1[a] (n = 4)	Week 2[a] (n = 4)	Week 3[a] (n = 4)	Week 4[a] (n = 4)	Week 5[a] (n = 4)
Left inguinal lymph node	0	0	0	0	1 (25%)
Right inguinal lymph node	0	0	0	0	1 (25%)
Left palatine tonsil	0/3	0	0	0/2	0
Right palatine tonsil	0	0/3	0	1 (25%)	0/3
Left mandibular lymph node	0/2	0	0	1 (25%)	0
Right mandibular lymph node	0/3	0/3	0	1 (25%)	0
Left parotid lymph node	0	0/3	0	1/3 (33%)	1 (25%)
Right parotid lymph node	0/3	0	0	0	0/3
Left deep cervical lymph node	0	0	0	1 (25%)	0
Right deep cervical lymph node	0	0	0	1 (25%)	0
Left cranial lung lobe	0	0	3 (75%)	4 (100%)	3 (75%)
Left caudal lung lobe	0	0	2 (50%)	4 (100%)	4 (100%)
Right cranial lung lobe	0	0	2 (50%)	2 (50%)	4 (100%)
Right middle lung lobe	0	0	2 (50%)	4 (100%)	3 (75%)
Right caudal lung lobe	0	1 (25%)	1 (25%)	4 (100%)	4 (100%)
Right accessory lung lobe[b]	-	-	-	2/2 (100%)	3/3 (100%)
Left anterior mediastinal lymph node	0	0	4 (100%)	4 (100%)	4 (100%)
Right anterior mediastinal lymph node	0/3	0/3	4 (100%)	4 (100%)	4 (100%)
Mesenteric lymph node	0	0	1 (25%)	1 (25%)	1 (25%)
Gastric lymph node	0	0	0	1 (25%)	0
Hepatic lymph node	0	0	0	2 (50%)	3 (75%)
Liver	0	0	0	3 (75%)	2 (50%)
Spleen	0	0	0	1 (25%)	2 (50%)
Left kidney	0	0	0	0	0
Right kidney	0	0	0	1 (25%)	0
Right adrenal gland	0	0	0	0	1 (25%)
Thymus	0	0/2	0/3	1/3 (33%)	0/2

[a]The denominator refers to the number of observations where samples were not examined from all animals at that time.
[b]As the right accessory lung lobe was unavailable for histopathological examination, the data relate to macroscopic findings only.

THE ENDO-BRONCHIAL ROUTE

In the lung, the earliest lesions (2 weeks p.i.) consisted of thickening of alveolar septa with macrophages and lymphocytes, and aggregates of similar cells in associated alveolar spaces (Figure 4.1). Neutrophils were occasionally observed, and the density of AFOs was also low. However, using a Least Significant Difference (LSD) test, the number or density of AFOs in lung lesions (Table 4.7) did not differ significantly with time ($p = 0.5272$).

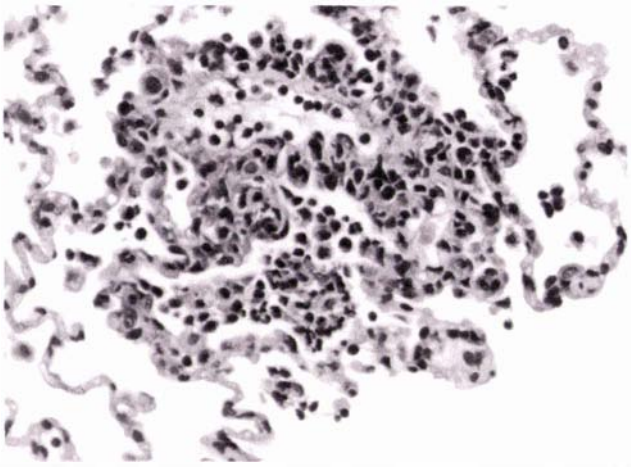

Figure 4.1 Early tuberculous lesion in the lung 2 weeks after endo-bronchial inoculation. Macrophages and lymphocytes surround a small blood vessel, and have infiltrated alveolar spaces and septa. H&E. Magnification = 235x.

Table 4.7 Comparison between bacterial counts from the right accessory lobe of the lung and numbers of acid fast organisms observed in the largest lung lesion from any lobe in histological sections following endo-bronchial and aerosol challenge

	Possums infected via E/B route		Possums infected via aerosol	
Weeks after *M. bovis* challenge	Bacterial count from lung (cfu/g)	No. of AF●s in 10 squares	Bacterial count from lung (cfu/g)	Density of AF●s (grade)
2	1220	116	<5	0
	0	181	<5	0
	990	59	<5	0
	16000	34	10	1
	1140	242		
	0	338		
	0	110		
3	700000	74	<5	1
	800000	152	<5	12
	510	128	4500	2
	1060	313	40	6
	400000	155		
	330	282		
	350000	170		
	410	133		
4	18000	125	2270	16
	254000	48	<5	9
	50000000	161	<5	9
			240	6
5			42000	12
			79550	6
			4040	1
			15	16

E/B = endo-bronchial; cfu = colony forming units; AF●s = acid fast organisms.

Although perivascular lesions were seen in a small number of possums 2 weeks p.i., by week 3 perivascular lesions were common with arteries, arterioles and, less commonly, veins affected. In early lesions, these vessels were cuffed with macrophages which were in turn surrounded by a mantle of lymphocytes (Figure 4.2). The inflammatory reaction sometimes also involved the blood vessel walls, producing a vasculitis and granulomatous perivasculitis. As the extent of vascular involvement increased, adjacent lymphatics became dilated, in association with accumulations of inflammatory cells within their lumina. Neutrophils were numerous in larger lesions by week 3, and the density of AFOs also increased. By week 4, almost all pulmonary lesions were in the form of perivascular cuffs, usually in association with dilated lymphatics containing moderate numbers of macrophages. Macrophages flanked peripherally by lymphocytes were also present in alveolar septa and spaces around the lymphatics (Figure 4.3). Expansive lesions had evidence of necrosis and/or caseation, and there was an up to 10-fold increase in the density of AFOs compared with the lesions seen at week 2 p.i.

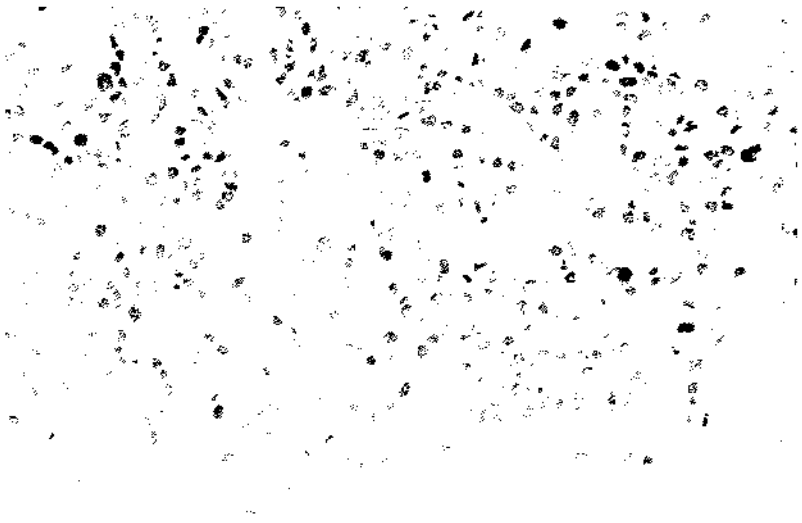

Figure 4.2 Lung of a possum 3 weeks after endo-bronchial inoculation. A distinctive aggregation of macrophages around a blood vessel, with lymphocytes peripherally. H&E. Maguification = 300x.

In anterior mediastinal lymph nodes at week 2, the earliest lesions, comprised solely of aggregates of angulated macrophages, were found predominantly in the cortex of the nodes. These were designated Stage 1 lesions (Table 4.8). Stage 2 lesions contained macrophages, were diffusely infiltrated with neutrophils (pyogranulomatous), and were evident at 3 weeks p.i., more commonly located in the paracortex than in the cortex. Stage 3 lesions consisted of pyogranulomatous lesions containing discrete foci of pyknotic cells and/or necrosis, and were the prevailing lesion by week 4. Stage 4 lesions showed the full spectrum of granulomatous inflammation, including areas of caseation. Early Stage 4 lesions, comprising a few small

caseous foci within areas of necrosis, were seen in nodal lesions in 63% of possums at 4 weeks p.i. The density of AFOs, most of which were intracellular, increased week by week, although extracellular AFOs were observed in areas of caseation. The bacteria were most numerous in the anterior mediastinal lymph nodes followed by the hepatic nodes.

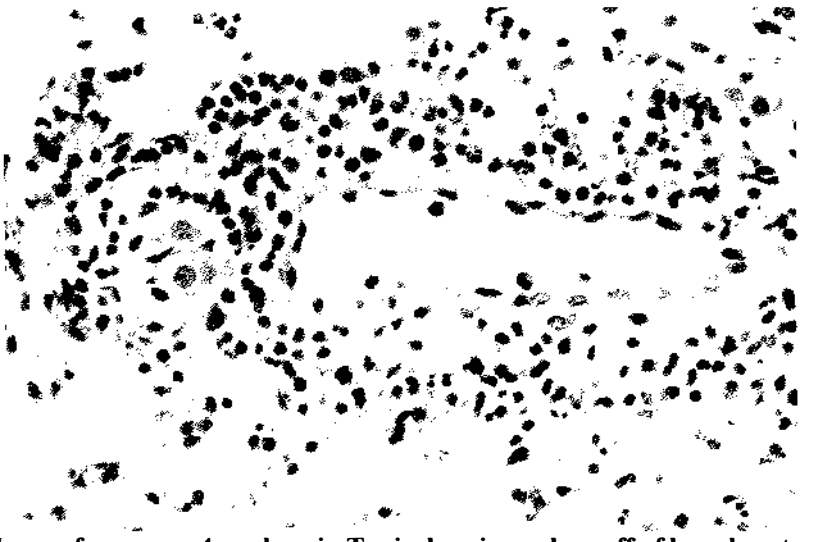

Figure 4.3 Lung of a possum 4 weeks p.i. Typical perivascular cuff of lymphocytes and macrophages. A small embolus of large plump macrophages may be seen inside a lymphatic vessel to the left of the blood vessel. H&E. Magnification = 300x.

Table 4.8 Dominant stage of histological lesions and assessment of the mean density score of acid fast organisms in lesions in the anterior mediastinal lymph nodes in possums following endo-bronchial and aerosol inoculation with *Mycobacterium bovis*

	Endo-bronchial inoculation		Aerosol inoculation	
Weeks after challenge	Lesion stage[a]	Av. AFO δ score	Lesion stage[a]	Av. AFO δ score
2	Stage 1	8	0	0
3	Stage 2	14	Stage 1	10
4	Stage 3[b]	30	Stage 2	12
5	N/A	N/A	Stage 3[c]	20

Av. = average (mean); AFO = acid fast organism; δ = density; N/A = not applicable.
[a]Stage 1 = macrophages.
Stage 2 = macrophages + neutrophils.
Stage 3 = macrophages + neutrophils + foci of pyknotic cells/necrosis.
Stage 4 = macrophages + neutrophils + foci of pyknotic cells/necrosis + areas of caseation.
[b]63% had small foci of caseation (i.e. early Stage 4).
[c]A few small foci of caseation were seen in all nodes (i.e. early Stage 4).

Liver lesions were randomly distributed in the hepatic parenchyma, and consisted of discrete small granulomatous foci composed of macrophages. Larger lesions were very occasionally seen, and were randomly infiltrated with low to moderate numbers of neutrophils. In the spleen,

granulomatous areas were located peripherally around periarteriolar lymphoid sheaths (PALS), and larger lesions involved the lymphoid follicles of PALS.

Lesions outside the lower respiratory tract were first seen in the liver and hepatic lymph nodes of one possum by 3 weeks p.i. (Table 4.5). Of all 10 possums at 4 weeks p.i., 30 sites were affected, ranging from 4-24 lesions per individual, with a mean of 12.1. After the lower respiratory tract, the liver and hepatic lymph nodes were the most commonly affected sites, followed by the spleen and the deep axillary lymph nodes.

LESIONS IN POSSUMS FOLLOWING AEROSOL INOCULATION

The nature of the lung lesions was very similar to that observed in the possums inoculated via the E/B route, with small lesions comprised of aggregates of macrophages centred on alveolar spaces and/or septa (Figure 4.4). Larger lesions were pyogranulomatous in nature (Figure 4.5), and more expansive lesions contained areas of necrosis and/or caseation (Figure 4.6). The density of AFOs also increased with increasing size and severity of lesions. Vasculitis and granulomatous perivasculitis were also observed, as was dilation of perivascular lymphatics.

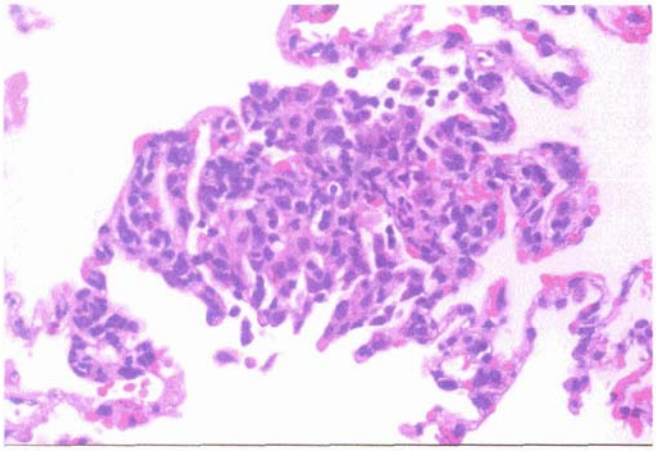

Figure 4.4 Early granulomatous lesion centred on an alveolar space at 2 weeks p.i.. Adjacent alveolar septa are thickened due to infiltration with mononuclear inflammatory cells. H&E. Magnification = 240x.

In the anterior mediastinal lymph nodes, aggregates of angulated macrophages (Stage 1 lesions) were found mostly in the paracortex and occasionally in the cortex, by week 3, and a few were occasionally variably infiltrated with neutrophils (Table 4.8). At week 4, the lesions were predominantly pyogranulomatous in nature (Stage 2), and small foci of degenerating neutrophils and pyknotic inflammatory cells were seen in a small number of lesions in two possums. Although more lesions were present in the cortex, the paracortex was predominantly affected. By week 5, there were large foci of necrosis (Stage 3), and occasional small foci of caseation

were seen within these aggregates. The LSD test showed that the density of AFOs at 2 weeks p.i.(LSD = 2.75) was significantly lower than the following weeks (at 5 weeks p.i. LSD = 10.75, p = 0.0103) (Table 4.7). The bacteria were most numerous in the anterior mediastinal lymph nodes followed by the hepatic nodes. The nature of the lesions in the hepatic parenchyma and spleen was similar to that observed in the possums inoculated via the E/B route.

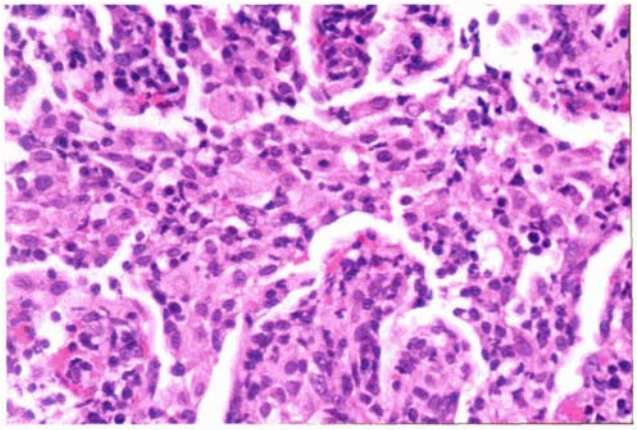

Figure 4.5 At 3 weeks p.i., this lesion is more advanced than Figure 4.4 and contains greater numbers of macrophages. A few neutrophils are also infiltrating the lesion. H&E. Magnification = 235x.

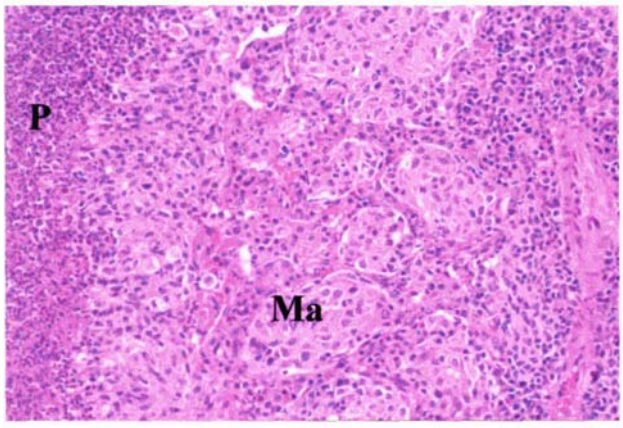

Figure 4.6 Expansive pulmonary lesion at 5 weeks p.i. A line of pyknotic inflammatory cells (P) may be seen to the left. Macrophages (Ma) fill alveolar spaces. H&E. Magnification = 115x.

Lesions outside the lower respiratory tract occurred in conjunction with pulmonary lesions and were first seen in the mesenteric lymph nodes of one possum by 3 weeks p.i. (Table 4.6). Lymph nodes of the head and neck were affected in one possum each by weeks 4 and 5, and the inguinal lymph nodes contained microscopic lesions in one possum after 5 weeks p.i. However, the most commonly affected sites after the lower respiratory tract were the liver and hepatic lymph nodes, followed by the spleen and the mesenteric lymph nodes.

4.3.3 Bacterial counts

Bacterial counts from the lung generally increased as the weeks p.i. increased (Table 4.7). There was a significant difference in the bacterial counts between 2 and 4 weeks following E/B inoculation (LSD = 6.14 cf. 14.33, p = 0.0498), and between weeks 2 and 5 following aerosol infection (LSD = 5.00 cf. 13.25, p = 0.0103). However, there was no correlation between bacterial counts from the lung and the numbers or density of AFOs recorded in histological sections, following both infection by the E/B route (Spearman's rank-correlation coefficient = -0.255, p = 0.306) and aerosol challenge (Spearman's rank-correlation coefficient = 0.304, p = 0.252).

4.3.4 Ultrastructural examination of alveolar macrophages

As the time after inoculation progressed, the number of AFOs in the alveolar macrophages increased, with AFOs first observed in the alveolar macrophages at 1 week p.i. (Figure 4.7). Activated alveolar macrophages were characterised by abundant cytoplasm, a ruffled exterior, and increased numbers of intracytoplasmic lysosomes and mitochondria. Bacilli were more commonly found free in the cytoplasm in the later stage of the disease.

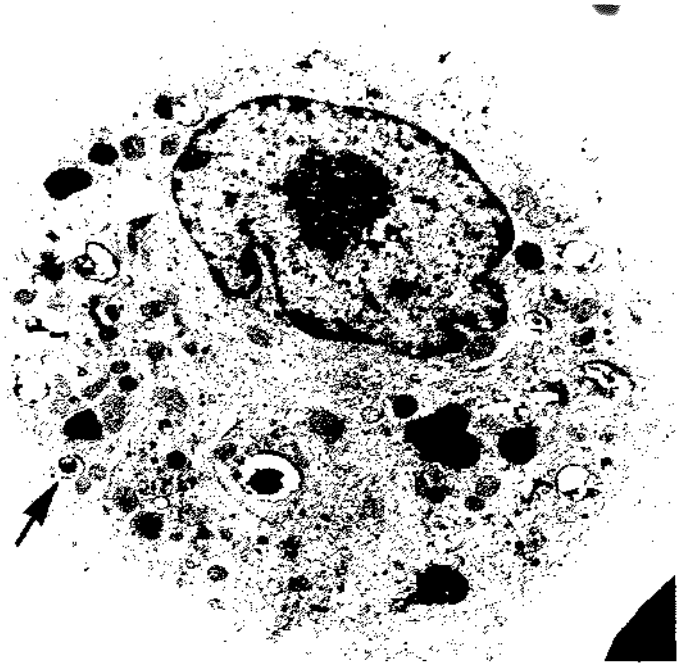

Figure 4.7 An activated alveolar macrophage with pseudopodia (1 week p.i.). Arrow indicates an intracytoplasmic *Mycobacterium bovis* bacillus. TEM. Uranyl acetate-lead citrate. Magnification = 7800x.

Although bacilli sometimes appeared to be contained within a vacuole (phagosome) (Figure 4.8), it was difficult to distinguish between bacilli free in the cytoplasm and those encased in tightly apposed vacuolar membranes. The features of tubercle bacilli enabling their identification were a nuclear region of low electron opacity surrounded by a high-density cytoplasm. This was bounded externally by a plasma membrane then a cell wall complex. An electron transparent zone was present between the bacillus and the phagosome wall.

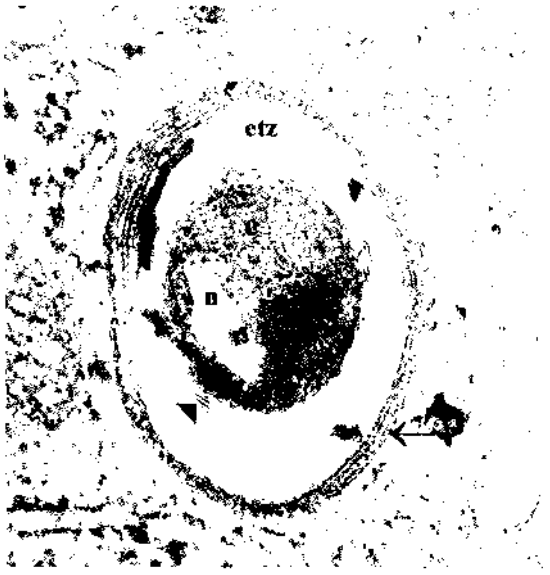

Figure 4.8 High power view of the *Mycobacterium bovis* bacillus in Figure 4.7. The bacterial cell wall complex is indicated by an arrow-head. The arrow indicates the phagosome wall. (etz = electron transparent zone; c = high-density cytoplasm; n = nuclear region). TEM. Uranyl acetate-lead citrate. Magnification = 103,600x.

4.4 DISCUSSION

Both methods of inoculation used in these experiments produced few macroscopic lesions outside the lower respiratory tract, despite the development of extensive pulmonary lesions in all the possums in the final week of infection. This differs from the natural disease, where extensive pulmonary lesions are invariably accompanied by large macroscopic lesions at other body sites. Out of the 53 possums inoculated by either method, only three (6%) had small macroscopic lesions in the liver, and these were not observed until 4 weeks p.i. Pulmonary consolidation occurred in most of the possums infected via the E/B route by 4 weeks p.i., whereas it was observed in only 19 of 117 (16%) cases of naturally occurring tuberculosis in non-terminally ill possums. Consolidation of the lung was observed in these natural cases of the disease, only in conjunction with large, often extensive lesions at many sites in the body, in particular, the liver, right kidney, and the mesenteric and hepatic lymph nodes (Chapter 3).

Although pulmonary consolidation was not observed in any of the possums infected via aerosol, pleural adhesions were evident in three of the four animals at 5 weeks p.i. In the natural disease, pleural adhesions were most commonly observed in terminally ill possums (Chapter 3). These differences in severity between experimental and natural infection could be explained by the likelihood that the natural disease is of a longer duration thus allowing more dissemination, in particular, seeding to the liver. Alternatively, the phenomenon of lymphatic blockade may have altered the progression and subsequent distribution of lesions by changing lymphatic flow and permeability. This phenomenon has been recognised in other mycobacterial diseases such as Johne's disease (paratuberculosis). It is believed that disordered lymph flow, which includes obstruction of lymphatics related to natural causes such as post-inflammatory conditions, and disease of vessel walls, renders the vessels incapable of uptake or retention of large molecules (Threefoot, 1977).

In the current experiments, few microscopic lesions were observed in peripheral lymph nodes, and in lymph nodes of the head and neck, when compared with the natural disease (Jackson *et al.*, 1995a). Only 7% of naturally infected possums with pulmonary tuberculosis did not have microscopic lesions in superficial lymph nodes (Chapter 3). Nevertheless, in the experiments presented here, there was a high frequency (70%) of microscopic lesions in the liver at 4 weeks p.i. Although this compares well with the natural disease, in which 65% of tuberculous possums had microscopic hepatic lesions, over 40% of the naturally infected possums had macroscopic lesions in the liver, while more than 40% had macroscopic lesions in other intra-abdominal organs (Chapter 3).

The propensity for microscopic lesions in hepatic nodes and high frequency of hepatic parenchymal Stage 1 lesions late in the course of the experimental disease is likely to be due to haematogenous and lymphatic spread. Microgranulomas are commonly observed in the liver of tuberculous ferrets (Lugton *et al.*, 1997b). Smith *et al.* (1970b) found that in sheep a large traffic of leucocytes passes from the blood to lymph through the liver and thus to the hepatic lymph nodes. Liver lesions would therefore not be unexpected in tuberculous animals, due to the high resident population of Küpffer cells, and macrophages draining from the liver to the regional nodes would account for the high frequency of lesions in hepatic lymph nodes. Microscopic lesions in the mesenteric lymph nodes, however, are likely to be due to lymphatic rather than haematogenous spread. In marsupials the mesenteric and mediastinal nodes receive lymphatic input from more than one region of the body. Azzali and Di Dio (1965) found that the right posterior cranial mediastinal lymph node in two marsupial species of *Didelphys* spp. receives efferent lymph from the stomach and diaphragm. Hopwood (1980) also found a

relationship in lymph flow between the cranial mediastinal lymph nodes and the abdomen in kangaroos.

As *M. bovis* has a propensity for lymphoid tissues, the lymph nodes draining the lung, namely the anterior mediastinal nodes, were selected for comparison of lesion development with affected lymph nodes in the natural disease. Full longitudinal sections of the anterior mediastinal nodes were easily examined, whereas it would be difficult to select a single lung lesion which would accurately reflect the development of the disease. A definite progression in lesion development associated with time was observed, ranging from granulomatous, through pyogranulomatous, to necrosis and caseation. Similarly, an increasing density of AFOs with increasing size and severity of lesions was demonstrated. In the natural disease, there was a comparable change in lesion morphology with increasing size of lesions, concomitant with an increasing density in AFOs, although the time of lesion development was not known. Thus, these experiments confirmed that small granulomatous lesions observed in naturally occurring tuberculosis in possums progress to become large pyogranulomatous lesions, followed later by necrosis and caseation, but the exact time scale for this development in the natural disease remains equivocal.

The lack of relationship between the numbers or density of AFOs in lesions and the bacterial counts was not surprising and may be due to a number of factors. In the histological sections, AFOs were assessed in the largest pulmonary lesions as these contained the highest density of organisms within each individual. Because this was a non-random selection, it may not have reflected the density of organisms in the lung as a whole. Furthermore, the thickness of the slides examined (4 μm), relative to the thickness of a tubercle bacillus (1 μm in width), allowed only a small proportion of the AFOs present in each lesion to be seen, and there was no evidence that the AFO distribution within a lesion was homogeneous. The bacterial counts were completed on cultures of the right accessory lung lobe. Macroscopic lesions were present in this lobe in only 25% of the possums examined, and the extent of these lesions was variable. Bacterial culture of the lungs indicated that spread of infection throughout the lung did not occur until 4-5 weeks after exposure to *M. bovis*. Cultural techniques using plate counts or plate dilution techniques are affected by the dispersion or otherwise of clumped bacteria (Collins *et al.*, 1974). Bacterial counts from one lung lobe only, are not therefore likely to be a suitable means of measuring the severity of infection in individuals, whether they are naturally or experimentally infected, except if conducted in the late stage of disease.

In both these experiments, there were important differences in the nature of pulmonary lesions from those seen in natural cases of tuberculosis. Lymphatic dilatation is not a feature of the

natural disease, and the vascular and perivascular lesions described in these experiments are uncommon in non-terminally ill possums in the natural disease. Nevertheless, vascular lesions do occur in possums which are terminally ill (Chapter 3). The differences between the natural and experimental infection may have resulted from variables such as the route and dose of infection employed, the volume of the inoculum, the virulence of the strain of *M. bovis* inoculated, or immunosuppression in the captive animals. Studies on the geographical distribution of tuberculosis in possums have established the presence of various localised strains of *M. bovis* (Collins *et al.*, 1986). It is possible that the strain used in these experiments may be of high virulence, and this may account for the rapid development and progression of lesions in the lung. Corner and Presidente (1981) found that a possum-adapted strain of *M. bovis* from New Zealand produced a more rapidly progressive and severe disease in experimentally infected possums than did an Australian cattle strain. The possibility that the stress of captivity induced immunosuppression cannot be discounted. Although every effort was made to reduce stress in the captive animals by habituation, there is still the possibility that changes in diet, human experimental and clinical manipulation, and close confinement of usually solitary animals may have resulted in immunosuppression.

It is known that inhaled tuberculous organisms preferentially colonise dorsal areas of the lung in most quadruped species, particularly cattle (Medlar, 1940). However, the results of the experimental aerosol infection of possums with *M. bovis* produced lesions that developed in all lobes of the lung. A similar distribution of lesions has been observed in the natural disease in possums. Previous studies (Chapter 3) have shown that after compensating for lung surface area and volume, pulmonary lesions in naturally occurring infections are randomly distributed throughout all lobes. This unusual distribution in possums may be due to factors such as mobilisation of the macrophage system probably takes an appreciable period of time, the anatomical structure of possum airways (Chapter 2), or more likely the marked variation in posture in relation to activity that is seen in this species.

In the animals infected via the E/B route, macroscopic lesions were first observed and were most prevalent in lobes of the right lung, in particular the middle and caudal lobes. As the animals were placed on their right side during inoculation, the combined effects of gravity and cannula length would account for this distribution of lesions. Wright (1961) stated that particulates introduced into the lung via liquid media (versus air) are distributed in a somewhat patchy manner, and the large concentrated dose usually overwhelms the normal defences, producing a quite different time course of subsequent events. Macroscopic pulmonary lesions in the possums infected via aerosol were spread more evenly throughout the six lung lobes. At 5 weeks p.i., aerosol infection resulted in macroscopic lung lesions more numerous and widely

distributed but similar in size and severity, as those produced by E/B inoculation at 3 weeks p.i. In general, the distribution of the pulmonary lesions following aerosol inoculation was similar to that seen in the naturally occurring disease.

The early experimentally induced lesions consisted of aggregates of macrophages in alveolar spaces and thickening of associated septa, and these probably arose from the localised deposition of the inoculum. By 4 weeks p.i. following E/B inoculation, pulmonary lesions were extensive, involving all lung lobes in most possums, due to embolic spread via the haematogenous and/or lymphatic routes, and from the aspiration of coughed respiratory secretions. Evidence of these mechanisms of intra-pulmonary spread was gained from histopathological examination of the left (contralateral) lobes of the lung, where lesions were centred in and around blood vessels, as well as in lymphatics adjacent to blood vessels, and were also centred on alveolar spaces and their associated septa. There was therefore strong histological evidence of rapid haematogenous and lymphatic spread very early in the course of intra-pulmonary *M. bovis* infection.

In earlier studies of exposure of possums to low doses of *M. bovis* (Pfeffer *et al.*, 1994), possums were killed 8 weeks after they were infected via the E/B route, by which time macroscopic lesions were extensive in both the respiratory tract and organs of the abdominal cavity. Pfeffer *et al.* (1994) found additional lesions, especially in lymph nodes of the head and neck and in peripheral lymph nodes. The presence of large numbers of bacilli in alveolar macrophages as demonstrated by TEM in the present experiments raises the possibility that lesions in lymph nodes of the head and neck may have been initiated by the presence of infected sputum in the oral cavity as early as 1 week p.i. The multifocal, vascular orientated nature of the pulmonary lesions produced by both methods of inoculation differs from the expansive lesions observed by Pfeffer *et al.* (1994), in which the duration of infection was twice that of the current experiment. The shorter duration and sequential examination of infected animals with time in the experiments presented here allowed the development of lesions to be followed.

A time delay of up to approximately 2 hours occurred between the collection of fresh lung at necropsy, and the harvesting of alveolar macrophages for TEM. However, specific criteria were used to identify tubercle bacilli (Armstrong and D'Arcy Hart, 1971), and it was still possible to identify the organisms inside many phagosomes. The processes involved in harvesting the alveolar macrophages, coupled with the time delay, produced artefact and resulted in bacilli less well preserved than expected. Tubercle bacilli multiply in unfused phagosomes, as survival of mycobacteria in cells is due to resistance to phagolysosome fusion (McDonough *et al.*, 1993).

In these studies, this corresponded with increasing densities of AFOs over time in association with increasing severity of lesions.

In the current experiments, peripheral blood lymphocyte blastogenic responses to *M. bovis* antigens were first detected at 3 weeks p.i., which was 1 week after lymphocyte infiltrations were detected in the lungs (Cooke *et al.*, 1999b). This finding suggests that there is sequestration of antigen-specific lymphocytes to the thoracic cavity as has been reported in tuberculous guinea pigs (*Cavia porcellus*) (Mainali and McMurray, 1998). This supports the theory of lymphocyte homing proposed by Yednock and Rosen (1989). These authors believe the homing specificities of some lymphocytes appear to be regulated by their history of antigen exposure as well as by the anatomical site in which antigen is encountered. An alternative hypothesis would be that in the early phase of the *M. bovis* infection, there is an enhanced local proliferation of lymphocytes in the bronchus-associated lymphoid tissue. However, the first detection of the lymphocyte blastogenic responses coincided with detection of macroscopic tuberculous lesions in the majority of the animals and occurred 1 week before the infections became generalised. Generalised infection was indicated by detection of microscopic lesions outside of the thoracic cavity.

In a previous experimental study of *M. bovis* infection in possums (Buddle *et al.*, 1994), the first appearance of peripheral blood lymphocyte blastogenic responses to *M. bovis* antigens coincided with the onset of adverse clinical signs, body weight loss, haematological changes and elevation of plasma fibrinogen. The onset of these changes was directly related to the number of *M. bovis* in the inoculum. Evidence from the current study would suggest that the presence of small localised tuberculous lesions in the lungs observed 2 weeks p.i. were insufficient to stimulate systemic cellular immune responses.

Aspiration of fine droplets containing only 1-3 tubercle bacilli each, of 1-4 μm in length, into alveolar spaces in experimental studies with rabbits may incite pulmonary tuberculosis (Wells, 1955). Despite the relatively low infective doses used in the current experiments, particularly that used in the possums infected by aerosol (10-20 viable *M. bovis* per possum), it is possible that the number of aspirated organisms was in excess of that which usually occurs in the natural disease. There are probably few situations in the natural disease where large numbers of organisms are likely to be aspirated.

Because there was a paucity of macroscopic lesions produced outside the respiratory tract, both experimental routes of infection used here are not ideal models of the natural disease. However, these results do contribute to a better understanding of the initiation and spread of pulmonary

tuberculosis in the possum. Infection via the E/B route produced extensive and severe lung lesions by 4 weeks p.i., very similar to that observed in the lungs of terminally ill possums. Even aerosol inoculation, where the infective dose was lower, more closely replicating natural pulmonary tuberculosis of possums, produced no macroscopic and few microscopic lesions in superficial lymph nodes, which is unlike the pattern of distribution observed in the natural disease. While these experiments highlight the susceptibility of the lung of the possum to aerosol infection with *M. bovis*, and demonstrate the rapidity of haematogenous and lymphatic spread, they fail to provide an explanation for the propensity of lesions in superficial lymph nodes. This suggests that another route of infection, such as through the skin, may also have a rôle in the pathogenesis of tuberculosis in possums.

CHAPTER 5. EXPERIMENTAL INOCULATIONS OF BCG VIA INTRA-DERMAL, ENDO-BRONCHIAL AND INTRA-VENOUS ROUTES

5.1 INTRODUCTION

In naturally occurring tuberculosis in possums, the number of animals with lesions involving peripheral lymph nodes exceeds those with lesions in the respiratory tract (Chapter 3). However, experimental infections of the respiratory tract with *M. bovis* have shown a paucity of lesions in the superficial lymph nodes (Chapter 4). This finding raises the possibility that an alternative route of infection, such as the skin, may also operate in the natural disease in possums. Early experimental studies involved inoculation of possums with *M. bovis* via a number of different routes including intra-peritoneal (I/P), intramuscular (I/M), oral (Bolliger and Bolliger, 1948); subcutaneous (S/C), and intra-nasal (I/N) (O'Hara *et al.*, 1976). The only previous experimental work using the intra-dermal (I/D) route of infection involved the administration of BCG to two possums (O'Hara *et al.*, 1976).

O'Hara *et al.* (1976) infected possums with possum and cattle isolates of *M. bovis*, via S/C and I/N routes. The S/C inoculations involved seven possums, which were injected with 0.2, 0.6 or 1.0 mg tubercle bacilli into the inner left thigh. All the injections resulted in abscesses at the site of inoculation, and the period of survival (25-100 days post inoculation (p.i.)) was dose dependent. Inoculation via the I/N route resulted in infection of regional (head) and mesenteric lymph nodes, and the lung in all four possums. However, the S/C inoculations resulted in a much wider distribution of lesions to those obtained by I/N inoculation. Additional sites affected, due to lymphatic and haematogenous spread, included axillary, inguinal and iliac lymph nodes, and adrenal glands.

Corner and Presidente (1980, 1981) conducted experiments using a cattle strain of *M. bovis* in Australia, and a second strain derived from tuberculous possums from New Zealand. Possums were inoculated via I/M injection in the left thigh, and all developed abscesses at the inoculation site by the time of euthanasia 2-10 weeks p.i. Doses used were 10^7 and 10^3 bacilli using the cattle strain, and 10^4 using the possum-adapted strain. In both sets of experiments, bacteraemia occurred by 2 weeks p.i., and the potential to shed bacteria from faeces, urine and tracheal exudate was demonstrated at 4 weeks p.i. Aerosol spread was only effected with the possum-

adapted strain of *M. bovis*, when both in-contact possums developed pulmonary tuberculosis, and one additionally developed generalised disease.

All these earlier experimental inoculations of possums used high doses of *M. bovis*, resulting in a rapidly fatal disease with widely disseminated lesions, typical of those seen in advanced or terminally ill cases of the natural disease. Most of the methods of delivery employed would be unlikely to occur in a natural situation. As a limited range of tissues was examined, frequently at macroscopic level only, and low numbers of animals were used, most of these experiments provided little or no information about the development or progression of lesions.

In order to better understand the pathogenesis of tuberculosis in possums, and to address the issue of lesions in peripheral nodes, a series of inoculation experiments using BCG was designed. As possums are highly susceptible to virulent *M. bovis*, BCG was chosen in the hope that the low virulence of the inoculum might mimic the response which occurs in the natural disease. In addition, the use of BCG reduced the need for strict biohazard control and minimised the risk of zoonosis. Possums were sourced from a non-endemic region for tuberculosis.

The main emphasis for the following experiments was the study of lesions produced in peripheral lymph nodes and visceral organs after inoculation via the I/D route. In case the lesion distribution seen following inoculation via the I/D route could be attributed to or complicated by infective material ingested during grooming of the inoculation site, a group of possums was also inoculated via the oral route. A third group of possums was inoculated via the endo-bronchial (E/B) route, as a means of comparison with both the natural disease and experiments using virulent *M. bovis*. A fourth group of animals was inoculated intravenously, to assess the lesion distribution produced by free (extra-cellular) organisms.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Seventy-seven possums (43 males and 34 females), weighing 1.6-3.4 kg, were captured in rural areas in the Manawatu region, New Zealand. The Manawatu region is a non-endemic area, where tuberculosis in possums is not known to exist. The animals were housed in individual wire cages (measuring either 60 x 50 x 40 cm or 50 x 40 x 47 cm) with a nesting-box

(measuring either 41 x 24 x 23 cm or 22 x 35 x 23 cm respectively) attached to the exterior of one side of the cage. The animals were given a diet of apples, oranges, and bread, with commercial possum pellets (Sharpes Grain and Seeds Limited, Lower Hutt) and water available *ad libitum.* Inoculation of possums followed a minimum period of 3 weeks acclimatisation designed to reduce the effects of stress caused by capture and housing (Buddle *et al.*, 1992). To facilitate handling for inoculation and euthanasia, possums were anaesthetised with an I/M injection of Ketamine HCl (Parnell Laboratories, New Zealand) at 25 mg/kg (Pfeffer *et al.*, 1994). Euthanasia was effected by intravenous (I/V) pentobarbitone injection.

5.2.2 Inoculum

The possums were inoculated with BCG-Pasteur 1173P2, grown to mid-log phase in 0.5% Tween albumin broth (Buddle *et al.*, 1997). The number of colony forming units (cfu) per millilitre (5×10^7) was determined by viable plate counts (Buddle *et al.*, 1994).

5.2.3 Experimental design

INOCULATION

Sixty-five possums were inoculated with 0.1 mL of BCG inoculum (i.e. 5×10^6 cfu per possum), and the remaining five were given 1.0 mL. Thirty-eight possums (22 males, 16 females) were inoculated by I/D injection into the dorsal midline of the neck, and seven (3 males, 4 females) into the dorso-lateral aspect of the left antebrachium, using a 26G needle. As it was not possible to deliver the total inoculum as one bolus, three or four injections at sites within 10 mm of each other, of a smaller volume were administered. Ten possums (5 males, 5 females) were inoculated via the oral route, via a 2 mm diameter cannula inserted per os deep into the oesophagus. Twelve possums (6 males, 6 females) were inoculated via the E/B route, via a 2 mm diameter cannula inserted through the mouth deep into the trachea (Pfeffer *et al.*, 1994). The animals were placed on their sternum while all the inoculum was flushed through the cannula with air. Ten minutes following inoculation, the possums were placed on their right side to recover. A further 10 possums (7 males, 3 females) were injected I/V into the right cephalic vein. Five of these animals were injected with 0.1 mL (low dose), and five with 1.0 mL (high dose) of inoculum.

DURATION OF EXPERIMENTS

Of the 38 possums inoculated via the I/D route into the neck, 13 were humanely killed at 1 week p.i., 12 at 2 weeks p.i., six at 3 weeks p.i., and seven at 4 weeks p.i. Three of the 10 possums infected orally were killed at 2 weeks p.i. The seven possums infected via the I/D route into the left antebrachium, seven of 10 possums orally infected, and the 10 possums infected I/V were all killed at 3 weeks p.i. For possums infected via the E/B route, five were killed at 2 weeks p.i., and seven at 3 weeks p.i. (Table 5.1).

Table 5.1 Experimental design and routes of inoculation with BCG

	Number of possums necropsied per week post inoculation (p.i.)				
Inoculation route	1 week p.i.	2 weeks p.i.	3 weeks p.i.	4 weeks p.i.	Total no. possums
I/D (neck)	13	12	6	7	38
I/D (foreleg)	-	-	7	-	7
Endo-bronchial	-	5	7	-	12
Intra-venous	-	-	10	-	10
Oral	-	3	7	-	10

I/D = intra-dermal.

5.2.4 Necropsy

The animals were necropsied and tissues from up to 40 separate body sites were collected for histopathological examination, using the methods described previously in Chapter 3. After fixation of the tissues in 10% neutral buffered formalin, the nature, size and distribution of any macroscopic lesions were recorded. All tissues were embedded in paraffin and routinely processed for the production of 4 μm thick sections stained by haematoxylin and eosin (H&E) and Ziehl-Neelsen[1] (ZN) stains.

A representative cross-section of skin, and the left deep axillary lymph nodes, were selected from the possums inoculated via the I/D route into the back of the neck. These tissues were used to stage lesion development according to time, for comparison with experimental *M. bovis* infections. Lesion types were divided into the four stages described in Chapter 4, and ranged from small granulomatous foci (Stage 1), through pyogranulomatous lesions (Stage 2), to lesions containing areas of necrosis (Stage 3) or caseation (Stage 4). The dominant stage of lesion was recorded for each week p.i.

[1]CullingCFA, Allison RT, Barr WT (eds). Cellular Pathology Technique. 4th Edtn. P 336. Butterworths, London, 1985.

The location of lesions in lymph nodes draining the skin of the neck and the antebrachium (i.e. the deep and superficial axillary lymph nodes respectively) were recorded from possums inoculated via the I/D route.

Assessment of the density of acid fast organisms (AFOs) in each possum inoculated via the I/D route into the back of the neck was performed on the most severe lesion in the skin and left deep axillary lymph nodes. The density of AFOs was assigned a score by assessing the number of macrophages containing AFOs, and the mean of 10 macrophages containing the highest numbers of AFOs, from a total of 100 macrophages examined. The average of the scores in the lesions was recorded for each week p.i.

The statistical method used was the Chi-squared (χ^2) test, analysed by the SAS (Statistix, Analytical Software Co., La Jolla, California) system computer programme.

5.2.5 Samples for electron microscopy

Samples of fresh tuberculous lung from seven of the possums inoculated via the E/B route, four at 2 weeks p.i. and three at 3 weeks p.i., were fixed in 3% gluteraldehyde. These were post-fixed in 1% osmium tetroxide in phosphate buffered saline (pH 7.4) for 1 hour and embedded in epoxy resin (Procure 812, Probing and Structure, Thuringowa, Queensland, Australia). Thin sections were cut and mounted on copper grids before staining with uranyl acetate and lead citrate, and the grids examined by transmission electron microscopy (TEM) (Philips 201c TEM).

5.2.6 Bacteriology

Half of the first two (the most cranial) deep axillary lymph nodes on both the left and right sides of the body were collected unpreserved for mycobacterial culture. These were taken from six animals each at 1, 2 and 3 weeks p.i., and seven animals at 4 weeks p.i. from possums inoculated via the I/D route into the neck. They were transported in a chilled state to AgResearch, Wallaceville, and were cultured by routine procedures (Buddle *et al.*, 1994).

5.3 RESULTS

5.3.1 Intra-dermal inoculation

Fourteen possums inoculated into the neck had visually detectable or palpable lesions at the site of inoculation (Figure 5.1). Eight of these were present at 2 weeks p.i., and two each were present at each of the remaining weeks p.i. The smallest lesions consisted of 1 mm diameter nodules covered by either small dry scabs or healed scars. These were detectable only by shaving the animals' hair to skin level, and were seen in four of the 13 possums at weeks 3 and 4 p.i. Palpable nodules ranged in size from 2 mm skin thickness through to the largest skin lesion; a pus-filled abscess of 10 mm in diameter. This lesion was the only one visible without shaving. Similar small scars were noted after shaving the forelegs of the animals inoculated into the left antebrachium. Apart from the lesions at the inoculation sites, no other macroscopic lesions were detected.

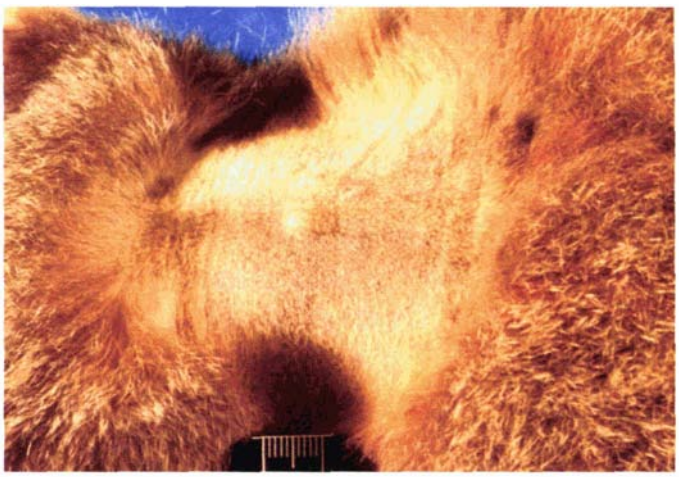

Figure 5.1 Swelling at the site of intra-dermal inoculation in the midline of the dorsum of the neck of a possum 2 weeks p.i.

Apart from inoculation site lesions, the most common site for microscopic lesions in the 38 possums inoculated via the I/D route into the neck was the right deep axillary lymph node, then the left deep axillary lymph node, followed by the hepatic lymph node. The distribution of histopathological lesions is given in Table 5.2 and Figure 5.2 (details are provided in Appendix XII). Seven possums had no detectable microscopic lesions, apart from those at the inoculation site. The only non-inoculation site lesions recorded at 1 week p.i. were in superficial lymph nodes. Lesions in the gastrointestinal tract (GIT) and spleen were found at 2 weeks p.i., and were very common at these sites by week 3 p.i. Microscopic lesions were detected in the lung

of only two possums, but were seen in the anterior mediastinal lymph nodes of eight possums. The greatest number of lesions was observed at 3 weeks p.i., with slightly fewer at 4 weeks p.i. The overall distribution of lesions at the five major body sites over the 4 week period was 76% in superficial lymph nodes, 26% in lymph nodes of the head and neck, 21% in the lower respiratory tract, 45% in the GIT, and 21% at other sites (Table 5.3). As seven possums had no microscopic lesions outside the inoculation site (Appendix XII), 29/31 (94%) of possums with non-inoculation site lesions had lesions in one or more of the six superficial lymph nodes.

Table 5.2 Distribution of microscopic lesions in possums killed at weekly intervals following intra-dermal inoculation with approximately 5 x 10^6 cfu of viable BCG into either the midline of the dorsum of the neck or the left antebrachium

	Number (%) of possums with microscopic lesions				
Lesion site	Week 1 (n = 13)	Week 2[a] (n = 12)	Week 3[a] (n = 6)	Week 3A[b] (n = 7)	Week 4[a] (n = 7)
Left superficial axillary lymph node	1 (8%)	0	4 (67%)	7 (100%)	0
Right superficial axillary lymph node	0	2 (17%)	2 (33%)	0	3 (43%)
Left deep axillary lymph node	1 (8%)	8 (67%)	4 (67%)	7 (100%)	4 (57%)
Right deep axillary lymph node	6 (46%)	10 (83%)	3 (50%)	1 (14%)	5 (71%)
Left inguinal lymph node	0	0	1 (17%)	2 (29%)	0
Right inguinal lymph node	0	2 (17%)	2 (33%)	2 (29%)	0
Right palatine tonsil	0	0	1 (17%)	0	0
Left mandibular lymph node	0	0	1/5 (20%)	0	1 (14%)
Left parotid lymph node	0	1/11 (9%)	1 (17%)	0	2 (29%)
Right parotid lymph node	0	1 (8%)	0	0	2/5 (40%)
Left superficial cervical lymph node	0	0	0	0	1 (14%)
Right superficial cervical lymph node	0	1/11 (9%)	1 (17%)	2 (29%)	1/6 (17%)
Left deep cervical lymph node	0	0	3 (50%)	0	1 (14%)
Right deep cervical lymph node	0	0	3 (50%)	0	0
Left cranial lung lobe	0	0	1 (17%)	0	0
Right cranial lung lobe	0	0	1 (17%)	0	0
Left anterior mediastinal lymph node	0	2/11 (18%)	1 (17%)	0	2 (29%)
Right anterior mediastinal lymph node	0	0	3 (50%)	0	2 (29%)
Mesenteric lymph node	0	3 (25%)	4 (67%)	3 (43%)	2 (29%)
Gastric lymph node	0	1 (8%)	3 (50%)	0	0
Hepatic lymph node	0	4 (33%)	6 (100%)	5 (71%)	5 (71%)
Liver	0	5 (42%)	3 (50%)	2 (29%)	1 (14%)
Spleen	0	3 (25%)	3 (50%)	1 (14%)	2 (29%)
Bone marrow	0	0	1 (17%)	0	0

[a]The denominator refers to the number of observations in cases where samples were not examined from all animals at that time.

[b]Possums at Week 3A were inoculated into the left antebrachium (the other four columns denote inoculation into the neck).

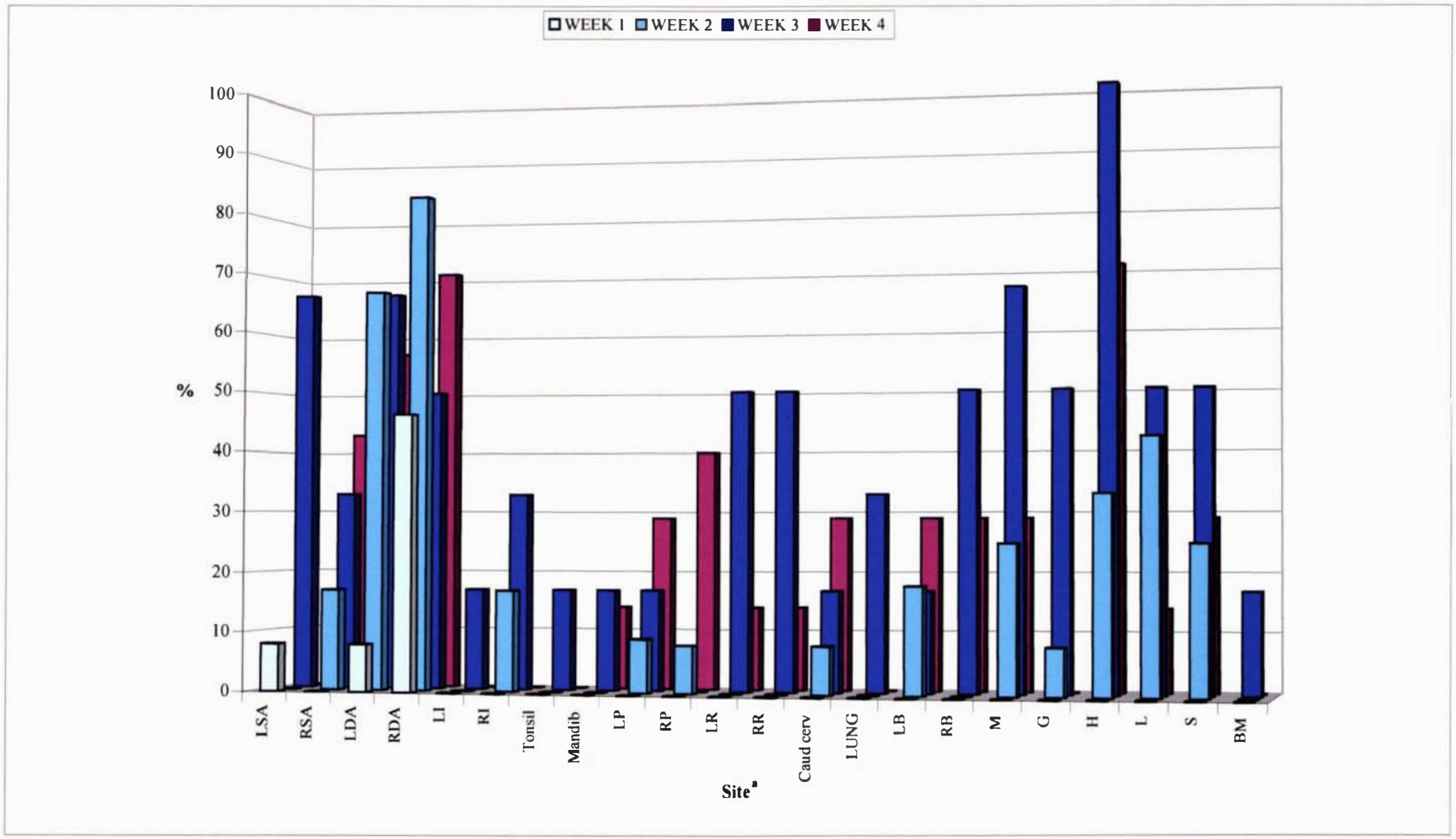

Figure 5.2 Distribution of microscopic lesions in 38 possums inoculated intra-dermally into the neck with BCG.

([a]LSA/RSA = left/right superficial axillary ln; LDA/RDA = left/right deep axillary ln; LI/RI = left/right inguinal ln; Tonsil = palatine tonsil; Mandib = mandibular ln; LP/RP = left/right parotid ln; LR/RR = left/right deep cervical ln; Caud cerv = superficial cervical ln; LUNG = all six lung lobes; LB/RB = left/right anterior mediastinal ln; M/G/H = mesenteric/gastric/hepatic ln; L = liver; S = spleen; BM = bone marrow; ln = lymph node).

Table 5.3 Total microscopic lesions at five major body sites in possums inoculated with BCG

Body site	Week 1	Week 2		Week 3				Week 4	Overall
Superficial ln[a]	54%	83%	0	100%	100%	29%	100%	86%	76%
Head and neck	0	17%	0	67%	29%	71%	100%	57%	26%
Resp[b]. tract	0	17%	100%	50%	0	71%	100%	43%	21%
GIT[c]	0	50%	0	100%	71%	29%	100%	71%	45%
Other sites	0	25%	0	50%	14%	0	100%	29%	21%

Intra-dermal (I/D) (neck); I/D (left antebrachium); Endo-bronchial; Intravenous.
[a]ln = lymph nodes; [b]Resp. = respiratory; [c]GIT = gastrointestinal tract.

All seven possums inoculated into the left antebrachium had microscopic lesions in the left deep and superficial lymph nodes. Outside the superficial lymph nodes, lesions were most common in hepatic lymph nodes, then mesenteric lymph nodes. Right-sided lesions occurred in three possums, and were in the deep axillary, inguinal and superficial cervical lymph nodes (Table 5.2 and Figure 5.3).

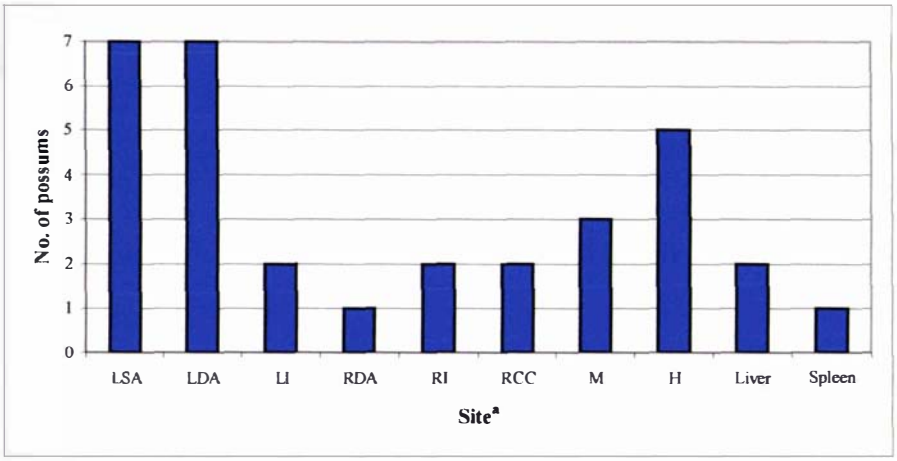

Figure 5.3 Distribution of microscopic lesions in seven possums killed at 3 weeks p.i. following intra-dermal inoculation into the left antebrachium with BCG.

([a]LSA = left superficial axillary lymph node (ln); LDA/RDA = left/right deep axillary ln; LI/RI = left/right inguinal ln; RCC = right superficial cervical ln; M/H = mesenteric/hepatic ln).

At both inoculation sites, the lesions in the skin were pyogranulomatous in nature (Stage 2), at all stages of the experiments. The lesions in the neck showed little variation throughout the duration of the experiment. However, pyknotic cells were seen in lesions in the neck (early Stage 3) in a third of the possums at 1 and 2 weeks p.i. (Table 5.4). In five possums, discrete subcutaneous lymphoid nodules were seen, and lymphatic spread was clearly evidenced by small granulomatous foci in one of these aggregates in one possum at 4 weeks p.i. (Figure 5.4). In contrast to the skin lesions, lesions in lymph nodes throughout the experiments consisted of small aggregates of angulated macrophages (Stage 1) (Figure 5.5). Despite a few neutrophils

being seen in a small number of these granulomatous foci, they were not generally a feature of lesions in lymph nodes.

Table 5.4 Predominant stage of histological lesions and assessment of the mean density score of acid fast organisms in lesions in the skin and left deep axillary lymph nodes of 38 possums inoculated via the intra-dermal route into the dorsum of the neck

	Skin		Lymph node	
Weeks after challenge	Lesion stage[a]	Av. AFO δ score	Lesion stage[a]	Av. AFO δ score
1	Stage 2[b]	23	Stage 1	1.0
2	Stage 2[c]	5	Stage 1	0.7
3	Stage 2	2	Stage 1	0.3
4	Stage 2	0.4	Stage 1	0.1

Av. = average (mean); δ = density.
[a]Stage 1 = macrophages.
Stage 2 = macrophages + neutrophils.
Stage 3 = macrophages + neutrophils + foci of pyknotic cells/necrosis.
Stage 4 = macrophages + neutrophils + foci of pyknotic cells/necrosis + areas of caseation.
[b]31% had small foci of pyknotic cells (i.e. early Stage 3).
[c]33% had small foci of pyknotic cells (i.e. early Stage 3).

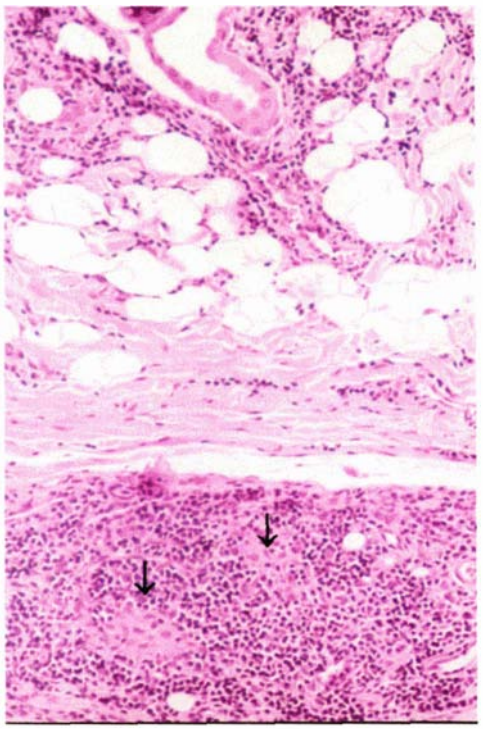

Figure 5.4 Small granulomatous foci (arrows) in a subcutaneous lymphoid aggregate adjacent to a pyogranulomatous lesion in the overlying dermis 4 weeks after intra-dermal inoculation with BCG into the neck. H&E. Magnification = 135x.

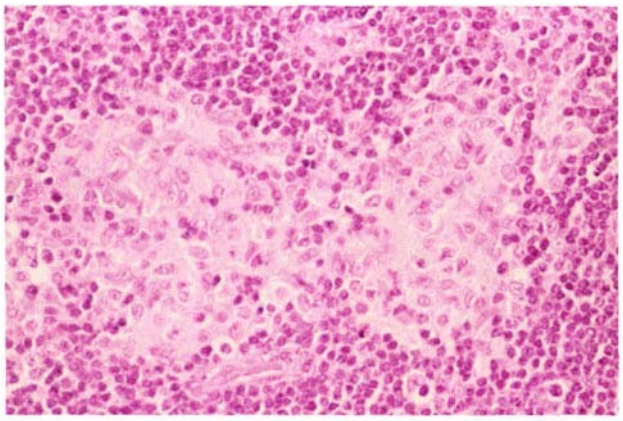

Figure 5.5 Small aggregates of angulated macrophages in an axillary lymph node of a possum 2 weeks p.i. with BCG via the intra-dermal route. H&E. Magnification = 230x.

With regard to both the deep and superficial axillary lymph nodes from possums inoculated into the neck and antebrachium (respectively), lesions were located in either the cortex or paracortex, in roughly equal proportions. Both inoculation sites produced a small number of lesions in the germinal centres of these lymph nodes.

Although there was relative uniformity in the morphology of lesions over time, the density of AFOs decreased with increasing time p.i. (Table 5.4). In skin lesions, the density of AFOs was highest at 1 week p.i., then dramatically reduced at 2 weeks p.i., and AFOs were seldom seen at 4 weeks p.i. Acid fast organisms in nodal lesions were increasingly difficult to visualise week by week, being sparse or non-existent by 4 weeks p.i., even though lesion morphology was still characteristic of that seen in tuberculosis in possums.

Of the deep axillary lymph nodes submitted for culture from 25 possums inoculated via the I/D route into the neck, 17 (68%) yielded *Mycobacterium bovis*. Negative cultures consisted of one animal at 1 week p.i., three animals at 3 weeks p.i., and four animals at 4 weeks p.i.

5.3.2 Endo-bronchial inoculation

At 2 weeks p.i., four of the five possums inoculated had macroscopic lesions in the respiratory tract. These consisted of small cream coloured nodules ranging in size from 1 to 9 mm in diameter, and were located in the left cranial and caudal lung lobes (two animals), and right middle lung lobe (two animals). One animal also had enlarged anterior mediastinal lymph nodes. Macroscopic lesions were detected at 3 weeks p.i. in the respiratory tract of three of the seven animals infected. All three had a small number of cream coloured nodules up to 5 mm in

diameter in the right middle lung lobe, one also had similar lesions in the right cranial lobe, and another had an enlarged right anterior mediastinal lymph node.

Microscopic lesions were confined to the respiratory tract at 2 weeks p.i. (Table 5.5). Lung lesions numbered from one to five or six per section, and were rarely seen in all six lung lobes. However, lesions were more common in the anterior mediastinal lymph nodes. Lesions were detected outside the respiratory tract at 3 weeks p.i., the most common site being the right deep cervical lymph node.

Table 5.5 Distribution of microscopic lesions in possums following endo-bronchial inoculation with BCG

	Number (%) of possums with microscopic lesions	
Lesion site	2 weeks p.i. (n = 5)	3 weeks p.i. (n = 7)
Right deep axillary lymph node	0	1 (14%)
Right inguinal lymph node	0	1 (14%)
Left palatine tonsil	0	1 (14%)
Left deep cervical lymph node	0	2 (29%)
Right deep cervical lymph node	0	4 (57%)
Left cranial lung lobe	2 (40%)	1 (14%)
Left caudal lung lobe	2 (40%)	2 (29%)
Right cranial lung lobe	2 (40%)	2 (29%)
Right middle lung lobe	3 (60%)	3 (43%)
Right caudal lung lobe	1 (20%)	1 (14%)
Right accessory lung lobe	1 (20%)	1 (14%)
Lung (total)	4 (80%)	3 (43%)
Left anterior mediastinal lymph node	3 (60%)	5 (71%)
Right anterior mediastinal lymph node	4 (80%)	5 (71%)
Respiratory tract (total)	5 (100%)	5 (71%)
Hepatic lymph node	0	2 (29%)

p.i. = post inoculation.

The majority of pulmonary lesions were pyogranulomatous in nature. They ranged from small focal accumulations of macrophages, to larger lesions containing moderate numbers of neutrophils, whereas neutrophils were only occasionally seen in lesions in lymph nodes. Lesions were located in alveolar spaces (Figure 5.6), frequently in association with type II cellular hyperplasia and thickening of alveolar septa with mononuclear inflammatory cells. Lesions were also observed around blood vessels, sometimes involving the vessel wall (Figure 5.7). In larger lesions, mixed inflammatory cells were present in the walls of some bronchioles.

Damage to the bronchiolar epithelium resulted in an exudate of mixed inflammatory cells which occluded the bronchiolar lumen (Figure 5.8).

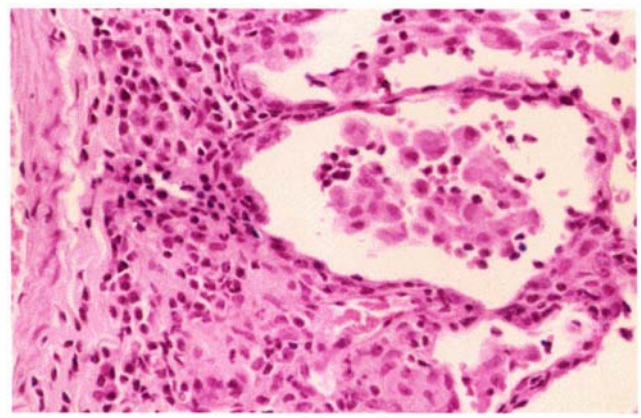

Figure 5.6 Lung from a possum inoculated with BCG via the endo-bronchial route 2 weeks p.i. Macrophages have accumulated in alveolar spaces and pyogranulomatous inflammation is present in the adjacent pulmonary parenchyma. H&E. Magnification = 235x.

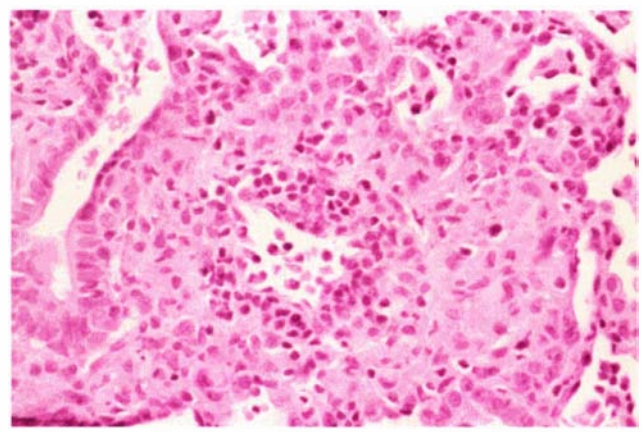

Figure 5.7 Granulomatous vasculitis and perivasculitis in the lung of a possum 2 weeks p.i. following endo-bronchial inoculation with BCG. H&E. Magnification = 235x.

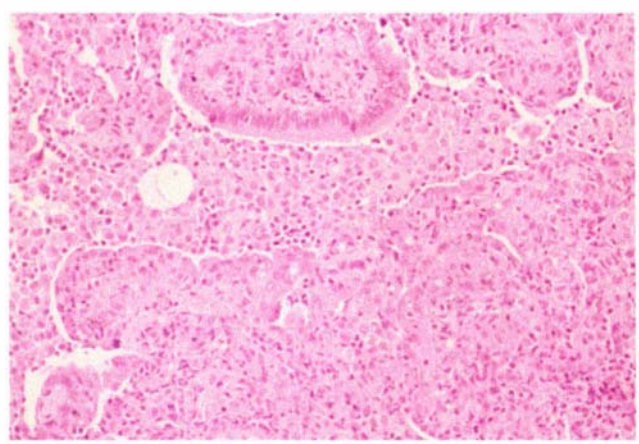

Figure 5.8 Extensive inflammatory exudate in the bronchiolar lumen of a possum 2 weeks p.i. with BCG via the endo-bronchial route. H&E. Magnification = 115x.

ULTRASTRUCTURAL STUDIES OF LUNG LESIONS

Within alveoli, the salient feature of E/B inoculation with BCG was type II cellular hyperplasia (Figure 5.9). These cells were enlarged and contained numerous characteristic dense and lipid secretory vacuoles in their cytoplasm. Cytosomes, comprised of osmiophilic lamellae, were located throughout the cytoplasm of type II cells, whereas in normal type II cells, they are primarily located on the luminal side of the cells. Because of the low numbers of BCG bacilli present by 2 weeks p.i., and even fewer by 3 weeks p.i., it was extremely difficult to differentiate the bacteria from the intra-cytoplasmic cytosomes in Type II cells and lamellated bodies in macrophages (Figure 5.10). Activated macrophages were recognised by their prominent pseudopodia, increased cellular size and protein content, and abundant cytoplasm. Cytoplasmic elements which increased in number included lysosomes, which were also larger than in non-activated macrophages, lipid vacuoles, mitochondria, myelin figures, and filaments. The few BCG bacteria identified were within intra-cytoplasmic phagocytotic vacuoles (Figure 5.11). Alveolar septal thickening was due to an influx of macrophages, and lesser numbers of other mononuclear inflammatory cells. Affected bronchioles were heavily infiltrated with macrophages, many of which were large and activated, and lesser numbers of neutrophils, lymphocytes, and occasional plasma cells.

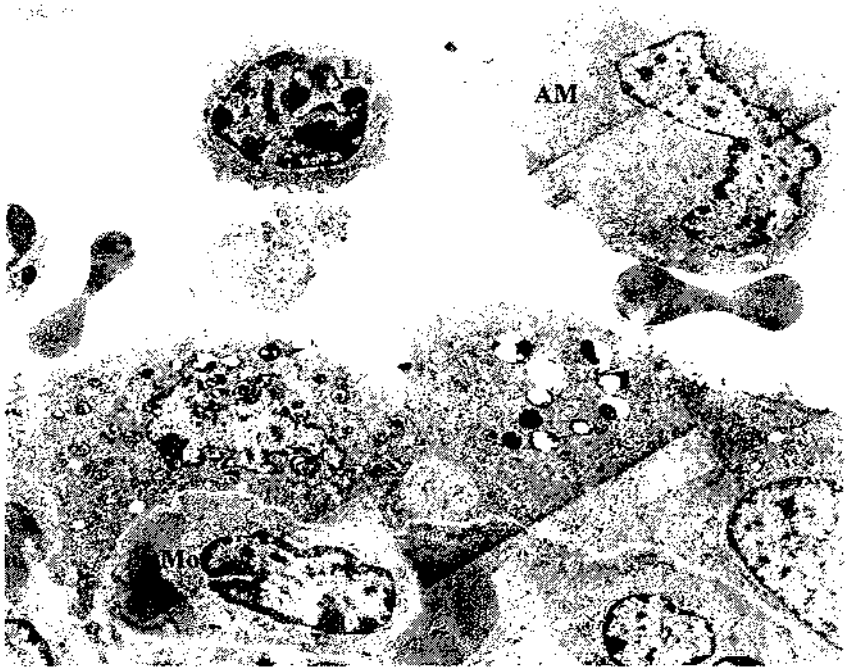

Figure 5.9 Type II cellular hyperplasia in the lung of a possum 2 weeks p.i. with BCG via the endo-bronchial route. Vacuolation is evident throughout the cytoplasm. A monocyte (Mo) may be seen inside a capillary. A lymphocyte (L) and alveolar macrophage (AM) lie free in the alveolar space. TEM. Uranyl acetate-lead citrate. Magnification = 7800x.

Figure 5.10 Intra-cytoplasmic dense vacuole with internal laminations in an alveolar macrophage in the lung of a possum 2 weeks following endo-bronchial inoculation with BCG. TEM. Uranyl acetate-lead citrate. Magnification = 103,600x.

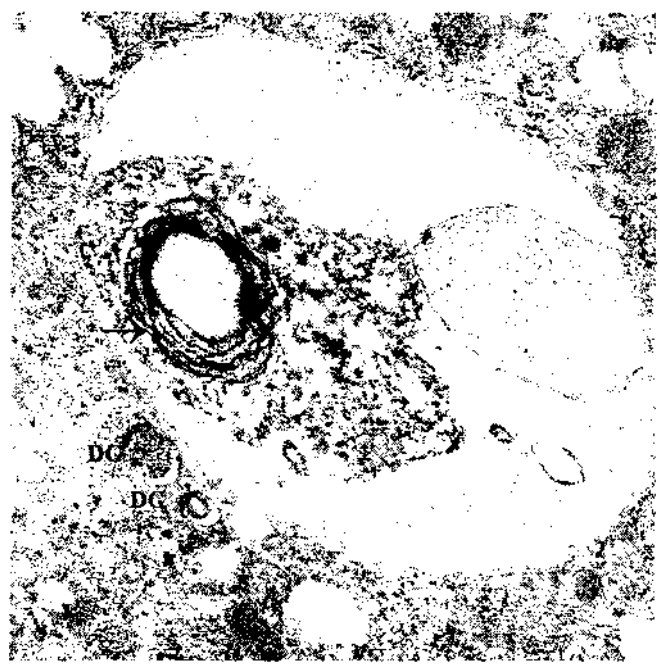

Figure 5.11 A presumed degenerating BCG bacillus (arrow) inside a phagocytotic vacuole of a macrophage. Dense granules (DG) abut the wall of the vacuole. TEM. Uranyl acetate-lead citrate. Magnification = 21,200x.

5.3.3 Intravenous inoculation

Although no macroscopic lesions were seen, almost all of the 40 sites examined contained microscopic lesions in all 10 possums (Figure 5.12). The superficial lymph nodes, respiratory

tract, GIT, spleen and bone marrow were affected in all possums, but other vascular organs such as the kidneys and adrenal glands were variably affected, with 40% of possums having lesions in the left or right kidney. In all eight of the possums from which the thymus was sampled, there was evidence of infection. The least affected sites were the duodenum and ileum (10% of possums), and colon (30%).

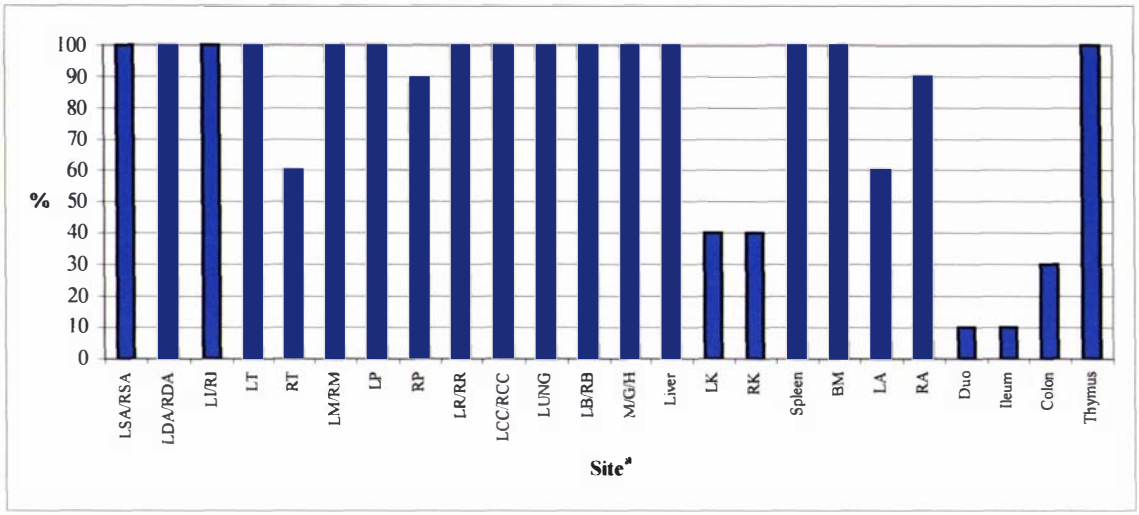

Figure 5.12 Distribution of microscopic lesions in 10 possums inoculated intravenously with BCG.

(*LSA/RSA = left/right superficial axillary ln; LDA/RDA = left/right deep axillary ln; LI/RI = left/right inguinal ln; LT/RT = left/right palatine tonsil; LM/RM = left/right mandibular ln; LP/RP = left/right parotid ln; LR/RR = left/right deep cervical ln; LCC/RCC = left/right superficial cervical ln; LUNG = all six lung lobes; LB/RB = left/right anterior mediastinal ln; M/G/H = mesenteric/gastric/hepatic ln; LK/RK = left/right kidney; BM = bone marrow; LA/RA = left/right adrenal gland; Duo = duodenum. ln = lymph node).

Most sites contained several microscopic lesions so that they were easily discernible even at low power examination. Lesions at most sites were pyogranulomatous in nature, and of moderate size. Necrosis and caseation were not observed, and there was an overall low to moderate density of AFOs. No difference in the size, nature or distribution of lesions was observed between the low dose (0.1 mL) and high dose (1.0 mL) groups of possums. Lesions in lymph nodes were found primarily in the paracortex. Those in the lung were centred round blood vessels, and in the spleen, lesions were adjacent to periarteriolar lymphoid sheaths (PALS). The majority of renal lesions were small and singular, consisting of a focal aggregate of macrophages in the cortical interstitium, which were difficult to see on low power. Although renal lesions were typical morphologically of tuberculous lesions in possums, none contained AFOs. Similarly, lesions in adrenal glands were predominantly granulomatous in nature, but there was usually more than one lesion present, and these were located in the cortex and at the cortico-medullary junction.

5.3.4 Oral inoculation

No macroscopic or microscopic lesions were detected in any of the animals inoculated orally.

5.4 DISCUSSION

The use of BCG produced microscopic lesions at many sites. Its low virulence was the most likely reason for few macroscopic lesions being produced, but this was useful for the purposes of this study as it ensured the animals were not overwhelmed by disease. Thus the early changes in the development and progression of tuberculosis could be observed. Experiments by other workers using BCG have also produced lesions. Legendre *et al.* (1979) inoculated cats subcutaneously with 0.25 mL of BCG, resulting in swelling and abscessation at the injection site, and a regional lymphadenopathy. O'Hara *et al.* (1976) injected two possums via the I/D route with 0.1 mL of BCG and recorded small nodular lesions in the lung of one and in the liver of both of the animals at 61 days p.i. However, it is unclear whether these lesions were confirmed by histopathological examination, raising the possibility they may have been due to other causes such as adiaspiromycosis or focal fatty change (Cooke *et al.*, 1995). Had the BCG experiments described here been allowed to run for a longer duration it seems unlikely that any more lesions would have become macroscopically visible, as lesions appeared to be resolving at 4 weeks p.i.

In the experiments described here, I/D inoculation resulted in a high frequency of lesions in deep axillary lymph nodes, similar to that observed in the natural disease. Lymphatic drainage studies in possums have shown that the deep axillary lymph nodes receive drainage from the superficial axillary and inguinal lymph nodes, and that all subcutaneous tissues drain either to the superficial cervical or the deep axillary lymph nodes (Jackson and Morris, 1996). The superficial cervical lymph node drains the skin of the cranioventral neck to the third cervical vertebra via the mandibular lymph node, whereas the deep axillary lymph node drains the skin of the dorsolateral neck and thorax. This has been confirmed by dye studies where drainage to the deep axillary lymph node occurred within 5 minutes after an I/D injection of Indian ink into the dorsum of the neck (pers. obs.). Researchers who clip the fur around the neck of captured possums have observed scars and scratches on the skin (B. Buddle, pers. comm.). Microscopic examination of macroscopically intact skin of the distal foreleg of two non-tuberculous possums revealed in one the presence of a foreign body (plant) granuloma in the dermis (pers. obs.). Depending on the site on the body's skin of initial entry of organisms, percutaneous infection of

possums would result in either the deep axillary or the superficial cervical lymph nodes being affected.

In the natural disease, lesions are more common in the left deep axillary than the left and right superficial axillary lymph nodes (Chapter 3). Although in the current experimental I/D inoculation into the neck there appeared to be more lesions in superficial lymph nodes on the right side of the body compared with the left, the difference was not statistically significant (χ^2= 1.52, p>0.2). However, a greater number of lesions occurred on the left side when injections were placed into the dermis of the left antebrachium. In this latter case, lesions were also prevalent in the left superficial axillary lymph node, as expected from knowledge of lymphatic drainage of the foreleg, and few lesions were recorded in the inguinal lymph nodes as both the I/D inoculation sites were in the upper half of the body.

Both sites of I/D inoculation produced lesions in the GIT of a similar frequency to that recorded in the natural disease (see Chapter 3). The hepatic lymph node was the most common GIT site for lesions, especially at week 3 p.i. In possums, this node drains the liver and the proximal duodenum (Jackson and Morris, 1996). Smith *et al.* (1970b) have shown that this node in sheep receives approximately 10-fold higher numbers of macrophages than other body nodes via the afferent lymph from the liver. Only about half as many lesions were recorded in lymph nodes of the head and neck, as well as "other" sites when compared with the natural disease. This finding may partly be due to the fact the experiments were of too short a duration to involve all lymph nodes, or, more likely, in the natural disease lesions in the head and neck nodes occur following upper respiratory tract infection.

Eight of 38 (21%) possums inoculated via the I/D route into the neck had microscopic lesions in the respiratory tract, but only two of these had lesions in the lung. This low frequency may be partly explained by the sampling technique used. The relative size of the possum's lung compared with the 4 μm thickness of histological slides would give a low chance of finding microscopic lesions, especially as only one section of each lung lobe was sampled. Fox (1928) states that the more widely distributed the tuberculous disease process is within the lung, the greater the lymph nodal involvement, but that the reverse is not the case, as there may be large caseous lymph nodes without discernable pulmonary parenchymatous foci. Once again, this highlights the likelihood that respiratory infection has a major rôle in the natural disease.

The small pulmonary lesions centred on alveoli produced by E/B inoculation with BCG were similar to the small pulmonary lesions seen in the natural disease. As in earlier experiments, the lesions remained largely confined to the lung, and the draining nodes were particularly affected.

In experiments using virulent *M. bovis* (Chapter 4), microscopic lesions were detected at a greater number of sites at 4 weeks p.i. than were detected at 3 weeks p.i. using BCG. But, when comparing lesion distribution at the same time interval of 3 weeks p.i, the patterns produced by BCG and *M. bovis* were very similar. Aerosol inoculation of possums with *M. bovis* produced a similar lesion distribution in peripheral lymph nodes and lymph nodes of the head and neck to that seen in the BCG inoculations via the E/B route (Chapter 4). However, aerosol inoculation produced lesions at more intra-abdominal sites than did E/B inoculation of BCG. Although lesions were detected in the peripheral lymph nodes of two (17%) possums at 3 weeks p.i. in the BCG E/B inoculations, there was an overall paucity of lesions at these and other body sites when compared with the natural disease. This may be a reflection of time, or low virulence of the BCG organism. Alternatively, the good housing and nutrition provided in the current experiments was less stressful than that used in previous experimental regimes, and may therefore have resulted in reduced immune suppression.

Evidence of haematogenous spread occurring early in the course of the disease was noted at 2 weeks p.i. following I/D inoculation into the neck. Lesions were recorded in lymph nodes of the head and neck, lower respiratory tract and GIT, as well as in the liver and spleen. Similarly, occurrence of lesions in lymph nodes on the (contralateral) right side of the body (deep axillary, inguinal and superficial cervical) in three possums following I/D inoculation into the left antebrachium could not be expected to occur from lymphatic spread. Haematogenous spread could have arisen from rapid extension into local subcutaneous blood vessels, which was commonly observed in the skin, or from rapid efferent lymphatic drainage into the bloodstream.

Following I/D inoculation into the neck, the lesions were most extensive at 3 weeks p.i. Similar observations have been made in other animals inoculated with BCG. Kindler *et al.* (1989) recorded the largest size and number of granulomas in the liver, spleen and lung of mice injected I/V with 1.2×10^6 cfu of BCG at 3 weeks p.i. Lurie (1932) inoculated rabbits with BCG into the lung and found severe lung pathology at 4 weeks p.i. In the current study, the 3 week optimal period for lesion development was also used when possums were inoculated via the oral and I/V routes.

It was speculated that following I/D inoculation of BCG into the midline of the dorsum of the neck, the animals would have difficulty grooming the skin at this site. To eliminate the possibility that infection could be due to the ingestion of tubercle bacilli from grooming, a group of possums was infected via the oral route. The dose of BCG administered orally was far in excess of that which the intra-dermally infected possums would have been able to accidentally ingest, yet no lesions were produced. Buddle *et al.* (1997) speculated that the low

pH of the possum's stomach could inhibit orally administered BCG. These authors produced enhanced immune responses and protection against bovine tuberculosis when the stomach was by-passed by intraduodenal vaccination of possums. Earlier work by Schwarting (1948) found that gastric contents inhibit the survival of mycobacteria in the stomach. These observations add weight to the hypothesis that the oral route is not important in the natural infection of possums with *M. bovis*. They also lend support to the theory that most lesions at GIT sites occur through haematogenous and/or lymphatic spread, or secondary to swallowed heavily infected sputum with subsequent establishment of lesions in the distal ileum (Patel and Abrahams, 1989; Corrin, 1990).

In the studies described here, I/V inoculation produced widespread miliary lesions at 3 weeks p.i., which, in lymph nodes, were located in the paracortex. In non-I/V routes of inoculation, lesions in lymph nodes at sites distant to the inoculation site were also located in the paracortex, supporting the likelihood that these were also haematogenous in origin. The organisms may arrive at lymph nodes within leucocytes migrating through blood vessel walls, or be taken up by resident macrophages in tissues, but in this latter case nodal lesions would not be expected to be present in the paracortex. Paracortical lesions may come about by sensitised T cells resident in the paracortex. These sensitised cells produce cytokines, which attract blood-borne macrophages to this area, and activate them. Because of the time intervals of lesion sampling in these studies, it may not be possible to distinguish whether paracortical lesions arose by haematogenous or lymphatic spread. However, in retrospect, a shorter time interval may have been useful in helping resolve this question, as well as better identify preferential sites for haematogenous spread.

The information gained from I/V inoculation of BCG allowed useful comparisons to be made with the natural disease, with regard to the distribution of lesions. The lesions produced intravenously were similar in distribution but not in magnitude to those seen in terminally ill possums (Chapter 3). This finding supports the hypothesis that in the final stages of the natural disease, there is a massive release of free organisms into the bloodstream. In terminally ill possums, AFOs were observed both intra- and extra-cellularly, the latter more commonly in areas of caseation. However, it is likely that the release of organisms into the bloodstream may involve the erosion of blood vessel walls (Patel and Abrahams, 1989).

The low frequency of renal lesions observed in possums infected intravenously with BCG is similar to that which occurs in the natural disease. Although renal lesions are sometimes seen in non-terminally ill possums, this only occurs when the disease is advanced (Chapter 3). Apart from the volume of blood perfused under pressure through renal tissue, other factors which may

affect mycobacterial uptake from the circulation are oxygen tension and the presence of resident macrophages. Skeletal muscle has a high oxygen tension and is seldom infected with *M. bovis*. In people, the most common sites for pulmonary lesions due to infection with *M. tuberculosis* are the apical lobes, which are the less well vascularised and ventilated areas of the lung. The liver receives a large volume of blood, yet microscopic lesions are not uncommonly found in this organ. However, the liver has a high resident population of Küpffer cells, which actively phagocytose foreign material in the bloodstream. Therefore, it seems likely that infection of any tissue with mycobacteria is related to the number of resident macrophages present; the kidney has a paucity of these cells. This organ is the predilection site for secondary lesions in tuberculous badgers (*Meles meles*) (Gallagher *et al.*, 1976), but the reason for this remains unexplained.

In these BCG experiments, there was a regression in lesion development over time, confirming the work of other authors. Kindler *et al.* (1989) infected mice via the I/V route with BCG, and recorded the largest granulomas at 3 weeks p.i., after which time the size of the granulomas declined slowly. Additionally, the BCG experiments showed that the density of AFOs in lesions decreased with time, as evidenced by histopathology, TEM and the bacterial counts. A similar reduction in the number of organisms after inoculation has been observed by other workers using BCG. Blanden *et al.* (1969) recorded the highest counts of BCG at 12 days following I/V inoculation of mice with BCG, after which the counts declined. When Lurie (1932) inoculated rabbits with BCG into the lung, he found a marked decrease in the number of AFOs by 2 weeks p.i. Buddle *et al.* (1997) vaccinated possums with BCG and noted a decline in the number of organisms after 2-7 weeks, until none were present at 13-16 weeks p.i. As would be expected, in both the natural disease, and in experimental respiratory tract inoculations using *M. bovis*, the density of AFOs increased with increasing size and severity of the lesions (Chapters 3 and 4). The decreasing AFO numbers are a reflection of the low virulence of BCG, and the ability of macrophages to phagocytose and kill them, as demonstrated by TEM, with eventual resolution of lesions. This confirms that BCG would not be useful in experiments of long duration.

In conclusion, these experiments demonstrated that I/D inoculation of BCG into the neck can produce a distribution of microscopic lesions similar to that recorded in naturally infected possums. The results suggest that lesions observed in deep axillary lymph nodes in the natural disease could originate from the entry of a small number of organisms through breaches in the skin around the neck. The notable exception to the similarity in pattern of lesion distribution produced following I/D inoculation with that of natural infections was in the lower respiratory tract, where only 21% of possums had lesions, compared with 79% of possums in the natural

disease. This confirms the principle that respiratory infection also has an important rôle in natural infection. This is further supported by the results obtained following inoculation of possums with BCG via the E/B route. The lesions produced in the lower respiratory tract in this case were consistent with those seen in natural *M. bovis* infections. There is, therefore, compelling evidence that in possums, both the skin and respiratory tract, infected either simultaneously or sequentially, are important routes of infection with *M. bovis*.

CHAPTER 6. GENERAL DISCUSSION

The study of naturally occurring tuberculosis in brushtail possums described here is the first detailed account of the pathology of the disease to date. It supports the experimental findings of Corner and Presidente (1980, 1981), Buddle *et al.* (1994), and Pfeffer *et al.* (1994), which demonstrated that the possum is highly susceptible to infection with *Mycobacterium bovis*. Evidence for this from the current study was provided by several observations. Few (17 of 117 (15%)) tuberculous possums had a single macroscopic lesion, a figure which reduced to only five (4.3%) at the microscopic level. Two thirds (78 of 117) of the possums had macroscopic lesions in two or more body sites (i.e. generalised disease), and assessment of macroscopic plus microscopic (total) lesions showed that 83% (97 of 117) had generalised disease. Until this study, pathological descriptions of lesions pertained to possums with advanced disease (Lake, 1975). This study has highlighted the expansive nature of tuberculous lesions in possums, and found that numbers of organisms increased in concert with increasing lesion size and development. Classical "tubercles" were not formed, and mineralisation, which commonly occurs in *M. bovis* infection in cattle and *M. tuberculosis* infection in humans, where infection is better contained, was a rare event, pointing perhaps to a rapid course of the disease. It is well recognised that a silent bacteraemia is a common early event in primary tuberculosis (Bates, 1980; Patel and Abrahams, 1989; Corrin 1990; Huchzermeyer *et al.*, 1994), but rarely causes overt disease. These latter authors stated that the bacillaemia occurs approximately 20 days after initial infection, and involves both bacteria in the blood, as well as infected macrophages entering blood and lymph vessels, ducts, or body cavities. In the work presented here, haematogenous spread was also shown to occur early in the course of the disease in possums, but in contrast with the situation in other mammals, bacteraemia resulted in the development of lesions, such as microgranulomas in the liver, at distant sites. Due to this high susceptibility to infection with *M. bovis*, it is probable that an extremely small number (one to three) of bacilli are required to initiate disease in the possum.

The detailed protocol implemented in the pathological study of tuberculosis in possums disclosed a two-fold increase in the number of lesions at the microscopic level compared with those noted macroscopically. Histopathology also had the advantage of affording the differential diagnosis of small lesions in organs such as the liver, lung and kidney. This methodology has now been successfully applied by other researchers to investigations of tuberculosis in possums (Corner, pers. comm.) and other species (Lugton *et al.*, 1997b).

The results of both the field and experimental studies undertaken have confirmed that the respiratory tract is an important route of infection in the possum. Houk (1980) stated that dried tuberculous secretions are very difficult to fragment and suspend in air, and any airborne particles which do arise from surfaces are usually innocuous as they are too large for lung deposition. This information suggests that there is little likelihood of infection of a possum sleeping with a tuberculous carcase. However, the possibility that the respiratory tract of the possum is poorly equipped to deal with this type of challenge cannot be discounted. As demonstrated in Chapter 2, the conventional mucociliary apparatus of the possum lung does not exist beyond the hilus of each lung lobe, and it is possible that not just droplet nuclei, but perhaps larger infective particles, may be able to reach the terminal airways and initiate infection. In the natural disease, pulmonary lesions were scattered widely and randomly throughout the lung (Chapter 3), whereas endo-bronchial (E/B) inoculation of *M. bovis* resulted in large localised lesions (Chapter 4), subsequent to the deposition of an infective bolus. Aerosol inoculation of possums with *M. bovis* used a low infective dose, but nevertheless produced rapid distension of blood vessels and lymphatics with macrophages around pulmonary parenchymal foci similar to that observed in terminally ill possums. This suggests that the dose delivered was still higher than that in the natural disease, even though the distribution of intra-pulmonary lesions was similar to that observed in naturally infected animals. Alternatively, the stress of captivity may have induced a state of immunosuppression in the experimentally infected possums, which may have rendered them more susceptible to low numbers of organisms and allowed their rapid dissemination.

Aerosols can be effective over short distances, and are likely to play an important part in the transmission of tuberculosis during simultaneous den-sharing. Extrapolating the experimental data of O'Hara *et al.* (1976), where tuberculosis was transmitted by aerosol between possums at a distance of up to 1.8 metres, Sauter and Morris (1995a) estimated transmission by aerosol was possible up to a distance of 1.5 metres (Figure 6.1).

The two major pathways for possum-to-possum spread of tuberculosis proposed by Morris and Pfeiffer (1995) are pseudo-vertical transmission and direct horizontal spread. The latter method occurs during courting and mating between sexes, and competition (fighting) among males, and epidemiological evidence strongly suggested that this takes place in areas around dens. Simultaneous den-sharing and grooming may make a minor contribution to direct transmission (Morris, 1994). Indirect horizontal spread is a minor pathway for transmission of tuberculosis between possums, and involves sequential den-sharing and chest-marking (Morris, 1994), whereas foraging and dispersal of (juvenile) males spread tuberculosis within and between possum populations (Coleman, 1988).

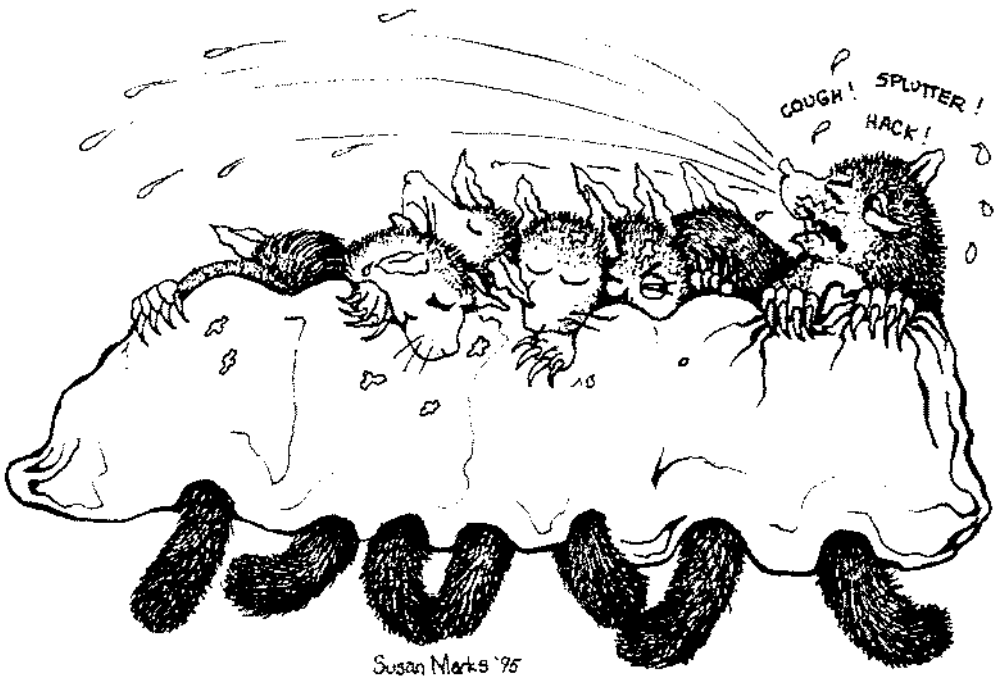

Figure 6.1 Possible means of transmission of tuberculosis between possums during instances of simultaneous den-sharing. (Reproduced with the kind permission of Susan Marks).

Pseudo-vertical transmission, which involves the close association between a female possum and her joey during suckling and grooming, is an important mechanism by which the disease is able to be maintained in a possum population so that contact with infected cattle is not necessary (Morris and Pfeiffer, 1995). Where the tuberculosis status of both dam and joey were known, 100% of joeys born to tuberculous mothers subsequently became clinically tuberculous (Morris *et al.*, 1993). Spread may occur from the respiratory tract, milk or discharging sinuses of the mother, infecting the joey by inhalation or ingestion.

At the microscopic level, significantly more naturally infected tuberculous possums had lesions in superficial lymph nodes than in the respiratory tract. Additionally, 14 of the 17 (82%) possums with single macroscopic lesions had them located in superficial lymph nodes, greatly outnumbering single macroscopic lesions at any other site. Thus, superficial lymph nodes are a predilection site for *M. bovis* deposition and development of lesions in the natural disease in brushtail possums.

Macrophages abound in lymphatic sinuses and the conditions of flow favour phagocytosis of particulate material carried into the node. If a lymph node fails to destroy infectious agents which they concentrate from the regions they drain, the node may serve as a new focus of infection and facilitate the spread of disease throughout the body. Lymphocytes leave the cortex of a lymph node via the lymph, move into the blood, and recirculate back to the

paracortex of lymph nodes of a similar anatomical site (Gowans and Knight, 1964; Chin and Hay, 1980; Joel and Chanana, 1987; Yednock and Rosen, 1989; Mackay *et al.*, 1990; Abitorabi *et al.*, 1996). However, Premier *et al.* (1996) recorded proliferative Th1-type memory T cells preferentially localising into the peripheral lymph nodes of sheep, independent of the site of antigen injection into sites drained by mucosal lymph nodes. Spencer and Hall (1984) found that lymphocytes from the caudal mediastinal lymph node of sheep entered the lymph from this node and peripheral lymph nodes with equal facility, but were less able to enter the intestinal lymph. Yednock and Rosen (1989) reported that paratracheal lymph nodes share characteristics of binding in both lung and peripheral lymph nodes, possibly because paratracheal lymph nodes receive combined lymphatic input from the lung and peritoneal cavity. Mesenteric and paratracheal lymph nodes receiving lymphatic input from more than one region of the body could provide overlapping spheres of regional immunity; antigen encounter within a hybrid node would result in simultaneous protection of multiple organ systems. Interpretation of pathology of lymph nodes of the thoracic cavity is dependent on examination of lymph nodes in both the thorax and the abdomen (Hopwood, 1980). Although lymphocytes are not actively phagocytic, they incite monocyte migration from the blood, and activate macrophages. It is possible that the lymph nodes of possums actively recruit cells containing phagocytosed organisms, although there is no evidence to support this theory.

Infection of superficial lymph nodes may be due to the retrograde flow of lymph, especially once lymphatics are occluded (Patel and Abrahams, 1989) in (severe) cases of pulmonary tuberculosis. Wood (1924) and Hopwood (1980) demonstrated the diversion of lymph flow in the opossum (*Didelphis virginianus*) and kangaroo (*Macropus* spp.) (respectively) by clamping a major efferent trunk. Later, Hopwood (1988) mentioned that although the inguino-axillary lymphatic pathway is of major importance in marsupials, it is quite possible that obstructive pathology could divert lymph into deeper lymphatic vessels. He believed that disease of superficial tissues of the hindlimb and tail may then result in consequential pathological change in both the axillary and iliac lymphocentres. Jackson and Morris (1996) speculated that lymphatic pathways may be altered by obstructive tuberculous lesions in lymph nodes, causing rerouting of lymph through collateral lymph vessels and unusual patterns of establishment of lesions in draining nodes. However, Innes (1940) reminds us that retrograde circulation of lymph and transport of bacilli is unlikely, as even the smallest lymph vessels possess valves, permitting flow of lymph in one direction only, and there is rich anastomosis of the afferent bed (from distant sources) of lymph nodes. He did concede that retrograde flow could occur but only if stasis is present over a very wide area.

The above explanations do not account for the discrepancy between lesions macroscopically evident in superficial lymph nodes in the natural disease and absent from the lung. Similarly, if infection of superficial lymph nodes is via the respiratory tract, then experimental respiratory *M. bovis* infections (Chapter 4) would have been expected to produce infection, particularly macroscopic lesions, in the superficial nodes. Yet, despite extensive pulmonary lesions, very few lesions were detected in these nodes, even at the microscopic level. Lymphatic blockade is therefore unlikely to satisfactorily account for the occurrence of lesions in superficial lymph nodes.

Since superficial lymph nodes drain the skin, infection of these nodes may reflect entry of tubercle bacilli through breaches in the skin. Indeed, the high proportion of lesions observed in superficial lymph nodes has already led some authors to speculate on the possibility of infection through the skin (Cook and Coleman, 1975; Hutton, 1979; Julian, 1981; Hickling *et al.*, 1991). Pfeffer *et al.* (1994) noted a paucity of lesions in peripheral lymph nodes in their experiments following E/B infection of possums, and mentioned that another route of infection, such as that involving wounds, might be responsible for lesions at these sites. Some authors have rejected percutaneous infection as a likely route of infection in possums, due to the lack of easily detectable skin lesions in field cases. In contrast, inoculation site abscesses have been recorded in experimental work (O'Hara *et al.*, 1976; Corner and Presidente, 1980, 1981; Buddle *et al.*, 1994), and in most instances were due to the very high challenge doses used. Morris (1994) thought it doubtful that superficial lymph node infection is due to infection through skin injuries. Buddle *et al.* (1994) concluded that in their experiments only a few viable organisms initiated the skin lesions, suggesting that possums are very susceptible to subcutaneous infection with *M. bovis*.

Despite their lack of enthusiasm for the skin as a route of infection in possums, few authors would refute the concept that *M. bovis* can infect wounds and scratches, and be cleared via lymphatics to the draining lymph nodes. Proof for this was provided from the current experiments involving intra-dermal (I/D) inoculation of BCG, which, in 86% of possums at 4 weeks post-inoculation, resulted in the development of lesions in superficial nodes draining the infected skin (Chapter 5). Apart from one macroscopically enlarged lesion, no inoculation sites would have been apparent without shaving the animals' necks. During post mortem examinations of possums, generally only markedly obvious skin lesions, such as torn ears, are detected, and it is likely that all macroscopic lesions of the type seen in the BCG experiments would escape detection. Furthermore, recent personal experience confirmed that inoculation site abscesses do not always develop, and that despite attempts to clean the wound, infection can become established at another site to which the bacteria drain (Appendix III).

Some of the problems associated with experimental inoculations of animals, especially wild animals such as possums, are raised by the current work. Regardless of the route of inoculation, it would be technically difficult to experimentally infect possums with only one to three tubercle bacilli, yet this may be the number of organisms which cause infection in the natural disease. Reducing the exposure time to *M. bovis* organisms could result in infection with fewer bacteria, similar to the natural situation. Eliminating sedation, and thus immobilisation, might prove helpful in future experiments using a very low dose of *M. bovis* via the I/D route, as this would allow possums the opportunity to groom the wound, reduce the chances of inoculation site lesions, and would more closely resemble the natural infection process.

Infective aerosols, and discharging sinuses, which were seen in 30% of tuberculous possums (Chapter 3), are likely to be the most common sources of infection. Histopathologically, all but the smallest lung lesion exhibited the potential to discharge infective material via the airways to the exterior , and intra- and extra-cellular organisms were seen on the surface of the skin around discharging sinuses. Electron microscopy confirmed the presence of viable *M. bovis* inside alveolar macrophages, particularly those obtained by bronchiolar lavage in experimental E/B infection with *M. bovis* (Chapter 4). Infective secretions coughed up from tuberculous pulmonary lesions would also contaminate saliva.

Skin breaches may occur during interactions between possums, or result from wounding on environmental objects such as sharp vegetation. Possums groom fastidiously, using saliva and the syndactylus claws which are combed through the fur (Biggins, 1984). They would groom discharging sinuses unless they were terminally ill, and thus their saliva would become contaminated during self-grooming. Contaminated saliva has the potential to infect wounds during intraspecific contact, including allo-grooming, which Day *et al.* (2000) noted is directed towards the dorsal part of the possum, or while self-grooming. Biggins (1984) reported that during mating, the male temporarily grasps the back of the female's neck with his teeth. He added that, once successfully mounted, the male clings to the female with his forelimbs circling her thorax or abdomen, and his hindlimbs clasp her hindlegs. Thus, infected claws and/or saliva could readily transfer infection to skin abrasions caused during copulation.

In his description of fighting between males, Biggins (1984) detailed grasping an opponent with forepaws and making lunging bites about the head, shoulders and neck. Although Paterson *et al.* (1995) found that 3% of nocturnal activity of possums on pasture was spent on other non-foraging activities such as threat and agonistic behaviour, no fights or close contact were recorded at that time, but subsequently Paterson (pers. comm.) observed fights between possums, and has found evidence of epilated fur on vegetation. In their review of the literature,

Day *et al.* (2000) reported that fights between possums are less common than threats, and high-intensity fights, during which much fur is lost and blood drawn from scratch and bite wounds, tend to occur between possums of similar dominance status.

Sources of *M. bovis* contamination of wounds are likely to be infective fomites, especially those inside dens, where organisms survive well in cool, dark, damp conditions. Areas where a number of tuberculous possums den together are high-risk locations for transmission of tuberculosis (Paterson *et al.*, 1995). The evidence that den-sharing occurs is now substantial (Anonymous, 1986; Fairweather *et al.*, 1987; Green and Coleman, 1987; Brockie, 1991; Pfeiffer and Morris, 1991; Caley *et al.*, 1998). Fairweather *et al.* (1987) recorded between three and five possums sharing dens with both living and dead possums. Caley *et al.* (1998) found up to four possums sharing a den together. However, Paterson *et al.* (1995) rarely observed simultaneous den-sharing, apart from that of mother-joey pairs. Pfeiffer and Morris (1991) observed a possum sleeping on three carcasses in one den.

Morris (1995) estimated that about 50% of transmission is from direct horizontal spread, 40% from pseudo-vertical transmission, and 10% from indirect infections. Yet, Day *et al.* (2000) reported that possums spend less than 1% of their time directly interacting, except during the breeding season, and Caley *et al.* (1998) estimated the highest daily probability of simultaneous den-sharing to be 7%. Thus, aside from the mother-young association, there are few incidences of intra-specific contact, and it would therefore seem more likely that indirect spread, such as sequential den-sharing, is more likely to be responsible for transmission between possums. Cowan (1989) reported that possums use 11-15 dens per year, and estimated there was about a 50% chance that a den would be occupied by different possums within the probable survival period of deposited tubercle bacilli. However, Morris *et al.* (1994) considered that surface contamination of den sites was not responsible for a significant amount of transmission among possums. Jackson (1995) proposed it highly unlikely that contaminated dens could feature in respiratory routes of infection, but refrained from discussing the issue of percutaneous infection, despite having evidence of tuberculous possums with peripheral lymph node lesions devoid of macroscopic lung lesions.

The presence of *M. bovis* in infected carcases is the most likely source of infection for scavenging or inquisitive animals, but putrefaction rapidly reduces the viability of the bacillus in decomposing carcases. The recovery of *M. bovis* from infected badger carcases on pasture (MAFF, 1979), and those which were buried (Little *et al.*, 1982a; Nolan and Wilesmith, 1994), has been variable, ranging from less than 4 days to up to 6 weeks. Pfeiffer and Morris (1991) had varying success in isolating *M. bovis* from decomposing possum carcases. They isolated

M. bovis from five of seven decomposed carcases of unknown tuberculosis status, on pasture, and a swab from three carcases found in a den also revealed the presence of *M. bovis*. Although they were unable to recover the organism from the dry carcase of a possum found 4 months after death, they did not specify that the animal was known to be tuberculous. In August 1993, Jackson *et al.* (1995a) were able to culture *M. bovis* from a partly autolysed possum carcase. It was unknown how long the carcase had been undergoing decomposition, but macroscopic tuberculous lesions were still detectable. Average weights of badgers vary according to sex, time of year, and food availability, but are approximately 10 kg (Neal, 1996), whereas possums weigh, on average, about 2-3 kg (Cowan, 1990). Although the number of organisms and the survival of *M. bovis* in a possum carcase might be expected to be considerably less than that recorded from badgers, the greater body mass of badgers could result in more rapid putrefaction and thus poorer survival of *M. bovis*.

To test environmental survival of *M. bovis* under New Zealand conditions, Jackson *et al.* (1995c) placed absorbent 3 cm strips of cotton ribbon impregnated with 3.5-16.0 x 10^5 *M. bovis* in dens used by possums, on the forest floor, and on open pasture. Bacterial survival was longest on samples placed in dens - 4 days in summer, 7 days in autumn, and 14 days in spring and winter – intermediate in forest floor samples, and shortest from samples on pasture. However, the medium selected for placement of suspensions of the organism was artificial, and survival rates may therefore be longer than those recorded.

Whilst percutaneous infection offers a good explanation for the presence of a proportion of lesions in superficial lymph nodes, it fails to account for the occurrence of pulmonary tuberculosis in animals with this route of infection, given that a paucity of lesions was produced in the respiratory tract following I/D inoculation with BCG (Chapter 5). More lung lesions may have been produced had the experiments run a few more weeks, but the phenomenon of lesion resolution with BCG infections precluded experiments of a longer duration. After 3 weeks, the I/V inoculation of BCG undertaken in Chapter 5 produced a distribution of lesions similar to that recorded in terminally ill possums. Therefore, I/V administration of BCG might prove useful in further experiments of shorter duration, in order to elucidate the preferential sites for uptake of haematogenous organisms, and the site of lesions of haematogenous origin in lymph nodes. Because extra-cellular bacteria commonly occur in advanced disease, particularly in liquefactive lesions (Dannenberg, 2000), this route of infection will not reflect the early stages of infection as much as the generalised disease.

To validate the occurrence of skin infection, subsequent to the experiments using BCG, a very low dose of *M. bovis* could be used to infect possums via the I/D route. This should reproduce a

similar pattern of lesion distribution to that observed in nature. Currently, it is not known what proportion of natural infections would be due to this means of infection. Extrapolation from the pathological findings in the field study, in which more than 20% of tuberculous possums had lesions in superficial lymph nodes but not in the respiratory tract, suggests that this may represent the natural frequency of infection via this route.

A relationship undoubtedly exists between both percutaneous and respiratory routes of infection, although it is not known what is the relative contribution of each route of infection. The pathological study demonstrated a correlation between discharging sinuses and pulmonary tuberculosis of all six lung lobes. Following haematogenous spread, skin infection may lead to respiratory infection, and vice versa. On one hand, superficial lymph nodes may become infected subsequent to the inoculation of a skin wound with contaminated saliva from a possum with pulmonary tuberculosis, or alternatively a possum may inhale infective droplets or particles while grooming a discharging sinus. It is also possible there may be more than one instance of exposure or encounter with tubercle bacilli for initiation of the disease.

Although significant advances have now been made in understanding the pathogenesis of *M. bovis* infection in possums, there are some areas which will require further work. From an epidemiological modelling viewpoint, the duration of the natural disease is important. But, without an adequate *in vivo* diagnostic test, such information remains elusive.

Appendix I. Papers by the author incorporated into this thesis

Appendix II. Related papers on tuberculosis written/contributed to by the author and referred to in this thesis

Refereed papers

Coleman JD, Cooke MM, Jackson J, Webster R. Temporal patterns in bovine tuberculosis in a brushtail possum population contiguous with infected cattle in the Ahaura Valley, Westland. New Zealand Veterinary Journal 47, 119-24, 1999.

Coleman JD, Jackson R, Cooke MM, Grueber L. Prevalence and spatial distribution of bovine tuberculosis in brushtail possums on a forest-scrub margin. New Zealand Veterinary Journal 42, 128-32, 1994.

Cooke MM, Jackson R, Coleman JD. Tuberculosis in a free-living brown hare (*Lepus europaeus occidentalis*). New Zealand Veterinary Journal 41, 144-6, 1993.

Jackson R, Cooke MM, Coleman JD, Morris RS. Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): I. An epidemiological analysis of lesion distribution. New Zealand Veterinary Journal 43, 306-14, 1995.

Jackson R, Cooke MM, Coleman JD, Morris RS, de Lisle GW, Yates GF. Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): III. Routes of infection and excretion. New Zealand Veterinary Journal 43, 322-7, 1995.

Non-refereed papers/reports

Coleman JD, Coleman MC, Cooke MM. Prevalence and spatial distribution of bovine tuberculosis in a possum population, Ahaura Valley, Westland. Forest-edge patterns: Year 4 - August 1995. Unpublished Landcare Research Contract Report: LC 9596/66. 14 pp. Landcare Research, Christchurch, 1996.

Coleman JD, Cooke MM. Prevalence and spatial distribution of bovine tuberculosis in a possum population, Ahaura Valley, Westland. Forest-edge patterns: Year 3 - August 1994. Unpublished Landcare Research Contract Report: LC 9495/66. 14 pp. Landcare Research. Christchurch, 1995.

Coleman JD, Cooke MM. Prevalence and spatial distribution of bovine tuberculosis in a possum population in the Ahaura Valley, Westland. Forest-edge patterns: Year 5 - August 1996. Unpublished Landcare Research Contract Report: LC 9697/61. 14 pp. Landcare Research, Christchurch, 1997.

Coleman JD, Cooke MM. Prevalence and spatial distribution of bovine tuberculosis in a possum population at Flagstaff Flat, Westland, in 1999. Unpublished Landcare Research Contract Report: LC 9900/113. 15 pp. Landcare Research, Christchurch, 2000.

Appendix III. Iatrogenic tenosynovitis of the author's forearm caused by *Mycobacterium bovis*

Introduction

Around 300-400 new cases of tuberculosis in humans are reported each year in New Zealand (Ministry of Health, 1996). In the 10 ½ year period from January 1985 to July 1995, 60 human cases of *M. bovis* infection occurred in New Zealand (Pooley, 1996). With both organisms, proportionately more Maori than Caucasians and more men than women are affected, and there is an association between increasing age and tuberculosis. About three quarters of *M. tuberculosis* (Ministry of Health, 1996) and *M. bovis* (Pooley, 1996) infections in humans are cases of pulmonary tuberculosis; common extra-pulmonary sites for both types of infection are the kidneys, lymph nodes, and joints/bones.

Clinical History

I am a right-handed Caucasian woman, aged 44, and was vaccinated with BCG at the age of 13-15 years. While conducting post mortem examinations on 143 possums, seven of which were tuberculous, that were trapped from a study site in Westland (Coleman and Cooke, 2000), two deep cuts were accidentally made in the fingers of the left hand. The lesions bled freely, and after the removal of gloves, were washed in a bucket of water containing 'Phensol'. Plasters were then applied. The cuts healed uneventfully, and no abscesses or chronic wounds were observed at their sites.

Clinical observations

Four months after the cuts occurred, the left hand and forearm developed an acute onset of unilateral pain and swelling of the digits, and the volar aspect of the wrist, causing restricted movement of the hand. The condition was more pronounced after a month, as it was not possible to fully close the hand or fully extend the fingers. Numbness of the middle and ring fingers (secondary carpal tunnel syndrome (CTS)) was also experienced. After a further month, the condition worsened. The left hand and wrist were severely painful, the hand and volar aspect of the wrist were markedly swollen, and the range of movement became more limited.

Within days of discontinuing a short course of high-dose steroids, the wrist became progressively swollen, and the pain interrupted sleep. Two to three weeks later, the medial aspect of the wrist appeared suddenly distended with fluid (Figure AIII.1).

Diagnostic investigations

Initial investigations included haematology and plain radiographs. A full blood count and differential were normal, and tests for rheumatoid factor, ANA and C-reactive protein were all

negative. The erythrocyte sedimentation rate (ESR) was 6 (normal = 0-15), and the level of uric acid was 0.25 mmol/L (normal = 0.12-0.42). The radiographs revealed soft tissue swelling, but no bony lesions or erosions, nor any evidence of degenerative joint disease. A tentative diagnosis of tenosynovitis was made, possibly associated with primary osteoarthritis, and causing median nerve compression (CTS).

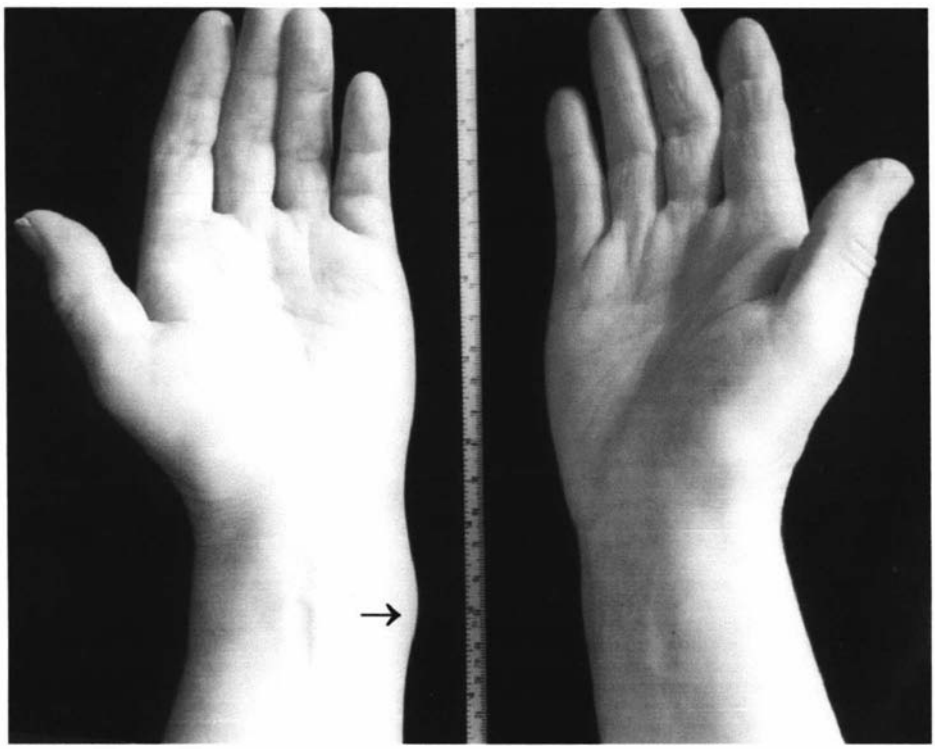

Figure AIII.1 Marked distension of the medial aspect of the left wrist (arrow), due to the accumulation of synovial fluid, and overall swelling of the left hand, in contrast with the unaffected right hand.

Radiographs taken a month later again showed marked soft tissue swelling but no changes to any bone or joint structures. Ultra-sound examination confirmed an accumulation of synovial fluid in the joint space of the left wrist, and magnetic resonance imaging revealed a florid tenosynovitis, extending from midway on the distal forearm to the digits. Using ultra-sound guidance, a fine needle aspirate of the synovial fluid and a needle biopsy of the synovium were collected for cytological and clinical biochemical evaluation, treated with KOH for the detection of fungal elements, and stained for the presence of micro-organisms including acid fast organisms (AFOs). The fluid was also cultured for micro-organisms, including AFOs. The fluid appeared clear and viscous. Cytology disclosed a reactive synovial membrane with an associated lymphocytosis, and there was no evidence of granulomas or malignancy, nor any presence of fungal elements and AFOs.

Four weeks after the collection of the synovial fluid, an acid fast bacillus had been cultured from the synovial fluid, and polymerase chain reaction (PCR) amplification detected the presence of *M. tuberculosis* complex DNA. Examination of the morphology of the colonies on agar plates suggested the most probable aetiological diagnosis was *M. bovis*, which was confirmed approximately 10 days later.

Histopathological examination of tissues collected during surgery and fixed in 10% formalin showed severe necrotising granulomatous inflammation. The inflammatory reaction was also present between muscle fibres. The synovial connective tissue contained numerous discrete and semi-discrete, coalescing granulomas, frequently centred on a large giant cell (Figure AIII.2). Each granuloma consisted of a wide mantle of macrophages, which was often surrounded by a thin band of lymphocytes, and peripherally there was evidence of fibrosis. A large area of acute, fibrinoid necrosis bordered an area in which fluid had been present, and this was thought to be due to rupture through the synovium. Although no AFOs were detected in sections stained by Ziehl-Neelsen, they were present in large numbers in the synovial fluid.

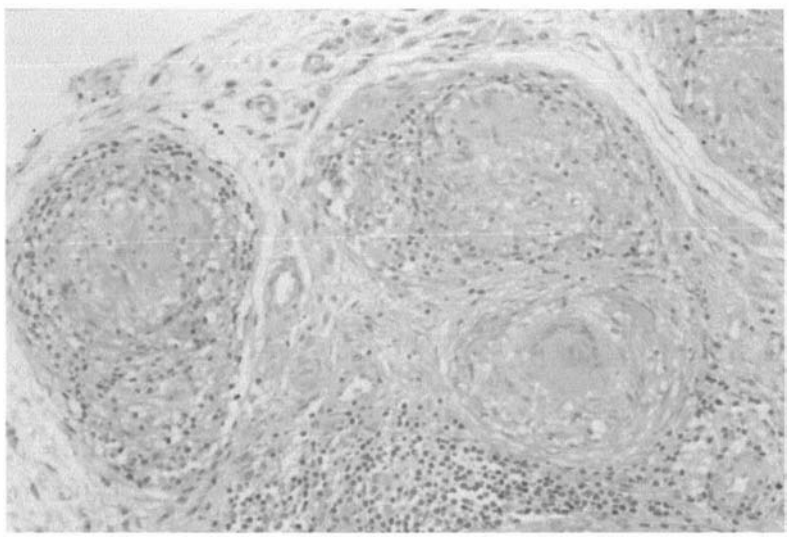

Figure AIII.2 Severe granulomatous tenosynovitis of the left wrist. Granulomas typically consisted of a large, centrally-placed giant cell, surrounded by a thick band of macrophages, encircled by lymphocytes, and enclosed by a thick band of fibrous tissue. H&E. Magnification = 145x.

Treatment

Following the tentative diagnosis of tenosynovitis and secondary CTS, the carpal tunnel was injected with 40 mg of Triamcinolone, with an improvement in symptoms expected to occur within the next 72 hours. The symptoms did not settle with the steroid injection, nor with oral 'Celebrex' (Celecoxib), a new cox-2 inhibitor anti-inflammatory drug. A month later, a short

course (12 days) of high dose steroids (Prednisone at 60 mg/day) exacerbated the symptoms, with noticeable swelling of the wrist, but eased the pain associated with the secondary CTS.

Three-and-a-half months after the initial onset of clinical signs, the wrist was surgically debrided under general anaesthetic, and the carpal tunnel decompressed. A large soft mass of thickened synovium, with an aggregate measurement of 50 x 40 x 25 mm was removed and submitted for histopathological examination. The tendon sheaths were stripped, and the incision closed. At removal of the sutures 13 days later, the surgical site was healing uneventfully (Figure AIII.3).

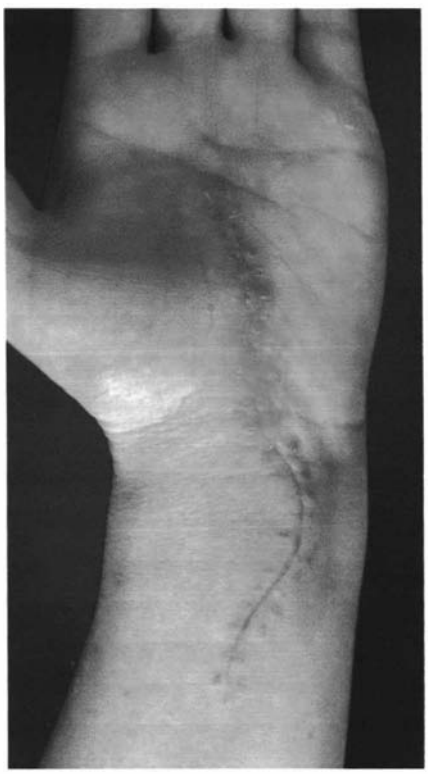

Figure AIII.3 Volar aspect of the left wrist following removal of sutures 13 days post-operative.

Six days after surgery, a 9-month course of triple anti-tuberculosis therapy – 'Isoniazid' (300 mg/day), 'Rifampicin' (600 mg/day), and 'Ethambutol' (25 mg/kg/day for 2 months) - was commenced. The delay between surgery and the start of chemotherapy was desirable for three reasons: the concomitant use of 'Rifampicin' and halothane should be avoided; the thrombocytopaenic action of 'Rifampicin' contraindicated its use immediately following extensive surgery; and commencing treatment at the beginning of a week facilitated access to support personnel in the case of adverse drug reactions. It is believed that the regime of chemotherapy, in conjunction with the surgery, will be curative.

Discussion and conclusions

Only two other cases of tenosynovitis caused by *M. bovis* have been reported. The first was that of a 45-year-old woman who pricked her right index finger while cleaning pipettes used for BCG cultures (Janier *et al.*, 1982). The affected finger was swollen for approximately two months prior to the development of CTS in the right wrist. Two corticosteroid injections were unsuccessful in treating the CTS, and were followed 5 months later by surgical decompression and exploration of the lesion. Histological examination of a biopsy collected during the exploratory operation revealed a necrotising, granulomatous tenosynovitis, and the presence of numerous AFOs. A discharging sinus developed at the surgical site, and a full recovery was made after 12 months triple anti-tuberculosis therapy. The authors advised of the dangers associated with the use of corticosteroid injections in cases of tuberculous tenosynovitis.

The second case of tuberculous tenosynovitis occurred in a 23-year-old, right-handed butcher, who had punctured his left wrist while slaughtering cattle (Bagatur and Bayramiçli, 1996). He presented with chronic tenosynovitis and secondary CTS, and similar clinical observations observed and investigations conducted as outlined in this recent case. A combination of surgical debridement and chemotherapy was curative.

In the case reported here, *M. bovis* was inoculated through the skin during the necropsies performed on tuberculous possums. Despite efforts to clean and disinfect the wound, organisms inoculated into at least one cut finger drained to the synovial fluid and around the tendons of the left wrist, where they became established and commenced to multiply. Although it is not known how many organisms were inoculated percutaneously, it is probable that only an extremely small number of viable organisms were involved. No tuberculous lesions were produced at the site(s) of inoculation. In retrospect, the corticosteroid injection (Janier *et al.*, 1982) and high dose oral steroids were contra-indicated, and the latter possibly resulted in rapid swelling of the wrist, due to multiplication of the organisms and extension of the lesion. Since ultra-sound is often used to break up clumps of tubercle bacilli commonly found in mycobacterial cultures (Brown, 1983), there is a possibility that the effects of the ultra-sound may have helped disperse the organisms by means of sonication.

People, such as diagnostic technicians, pathologists, butchers, trappers or hunters, and, to a lesser extent, farmers, who handle tuberculous animals and material are considered to comprise a high-risk group of contracting *M. bovis*. A greater risk of infection is predicted through aspiration of infective aerosols, than through skin infection, as the latter usually results in a localised, non-healing wound, which is unresponsive to conventional therapy.

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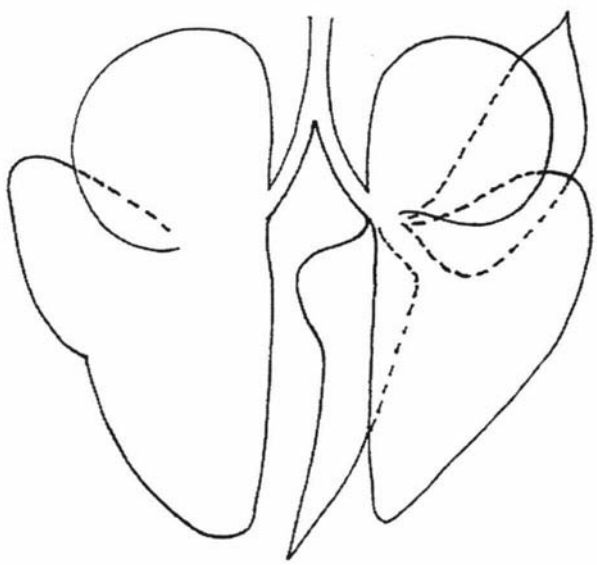
Appendix VI. Template for worksheets to record data obtained during necropsies and trimming of tissues

POSSUM NO.		DATE	
SEX	M F	AGE	IMMATURE MATURE
COLOUR	BL BR BB GR GB	LINE/SITE	
LACTATING	Y N	JOEY	Y N
JOEY'S SEX	M F	HEAD LENGTH	(mm)
BODY WEIGHT	(kg)	MESEN. FAT WT.	(g)
BODY LENGTH	(mm)	TESTES WIDTH	(mm)

LEFT	REMARKS	RIGHT	REMARKS
Super. axillary		Super. axillary	
Deep axillary		Deep axillary	
Inguinal		Inguinal	
Parotid		Parotid	
Mandibular		Mandibular	
Retropharyngeal		Retropharyngeal	
Caudal cervical		Caudal cervical	
Bronchial		Bronchial	
Mesenteric		Gastric	
Hepatic		Liver	
Kidney		Kidney	
Adrenal		Adrenal	
Spleen		Other	

C = Caseous
D = Discharging
E = Enlarged
N = Nodule
P = Pointing
Pp = Palpable



L R

Appendix VII. Template for waterproof paper for collection of lymph nodes and mammary glands

Left Super. Axillary		Right Super.Axillary	
Left Deep Axillary		Right Deep Axillary	
Left Inguinal		Right Inguinal	
Left Mandibular		Right Mandibular	
Left Parotid		Right Parotid	
Left Retropharyngeal		Right Retropharyngeal	
Left Caud-Cervical		Right Caud-Cervical	
Left Mammary Gland		Right Mammary Gland	
Gastric		Hepatic	
Mesenteric		Mesenteric	

Liver
Left & right Kidney
Left & right Adrenal
Spleen

Bone
Intestine
Jaw

Appendix VIII. Details of distribution of macroscopic and microscopic lesions in 117 tuberculous possums derived from field studies

Key

No.	tag number
LSA	left superficial axillary lymph node
RSA	right superficial axillary lymph node
LDA	left deep axillary lymph node
RDA	right deep axillary lymph node
LI	left inguinal lymph node
RI	right inguinal lymph node
LT	left palatine tonsil
RT	right palatine tonsil
LM	left mandibular lymph node
RM	right mandibular lymph node
LP	left parotid lymph node
RP	right parotid lymph node
LR	left (retropharyngeal) deep cervical lymph node
RR	right (retropharyngeal) deep cervical lymph node
LCC	left (caudal) superficial cervical lymph node
RCC	right (caudal) superficial cervical lymph node
LCR	left cranial lung lobe
LCD	left caudal lung lobe
RCR	right cranial lung lobe
RMD	right middle lung lobe
RCD	right caudal lung lobe
RAC	right accessory lung lobe
LB	left (bronchial) anterior mediastinal lymph node
RB	right (bronchial) anterior mediastinal lymph node
M	mesenteric lymph node
G	gastric lymph node
H	hepatic lymph node
L	liver
LK	left kidney
RK	right kidney
S	spleen
BM	bone marrow
LA	left adrenal gland
RA	right adrenal gland
Other	D = duodenum
	I = ileum
	C = colon
	LMM = left mammary gland
	RMM = right mammary gland
	2MM = left and right mammary glands
	T = thymus
Sex	F = female, M = male
Age	in years, or:
	I = immature
	M = mature
+	macroscopic tuberculosis lesion confirmed by histopathology and/or culture
d	discharging sinus
✓	microscopic lesion with acid fast organisms (AFOs)
(✓)	microscopic lesion characteristic of tuberculosis without AFOs
ns	not sampled

Appendix VIII. Details of distribution of macroscopic and microscopic lesions in 117 tuberculous possums derived from field studies (Page 1 of 6) – Page 179

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
B 6101		+		✓							ns		✓	(✓)					+ ✓	+ ✓	+ ✓		(✓)	✓	✓	✓	+ ✓	+ (✓)	+ (✓)	ns	+ ✓	(✓)				F	5
B 6102							✓	✓					✓	✓		ns					+ ✓			ns	+ ✓		✓			ns						M	2
B 6103	+		+	(✓)														+ ✓	+ ✓	+ ✓	+ ✓	✓	✓				✓		ns	+ ✓		ns				M	2
B 6104											ns	ns		ns											✓					ns						F	8
B 6106		✓	✓		+ ✓	(✓)		ns										+ ✓												ns		ns		ns		M	3
B 6110			+		+ d ✓		(✓)						✓				+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓			+ (✓)		ns			✓			M	2
B 6111	(✓)	+ ✓	✓	(✓)	+ d ✓	+		✓					✓				+ ✓	+ ✓	+ ✓		+ ✓	+ ✓	✓	(✓)	(✓)		✓		ns	+ ✓						M	8
B 6113					✓	(✓)																		ns						ns		ns				F	5
B 6114	+		✓			(✓)																									ns					M	1
B 6115	(✓)	(✓)	(✓)	✓	✓	✓			ns			ns	(✓)		ns	ns	+ ✓	+ ✓	+ ✓	+ (✓)	+ ✓	+ ✓			+ ✓			+ (✓)						✓		M	5
B 6117	✓	✓		✓			✓	✓						+ ✓	ns		+ ✓	+	+ ✓	+ ✓	+ ✓		+ ✓	✓	✓		✓	+ (✓)		ns	(✓)			ns		M	1
B 6118		+ d		+ ✓	✓	✓											+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓		+ ✓	+ (✓)	+ ✓	+ (✓)			✓	✓	RMM (✓)	F	3
B 6119					✓					✓		+		(✓)		✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓		+ ✓	+ ✓	+ ✓	+						M	3
B 6122	✓					(✓)											+ ✓	+ ✓		(✓)	(✓)		+ ✓	✓												F	5
B 6123	+ d	✓	✓	✓	(✓)				(✓)								+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓		ns	+ ✓								M	1
D 0218	+ d ✓	+ d ✓	+ ✓	✓	+	✓	✓	✓	(✓)	ns							+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)	✓	✓		✓	+ ✓	+ ✓		(✓)					M	1
D 0280	✓		+ ✓			✓						ns					✓	(✓)			✓			ns			✓	✓		ns	✓	(✓)		ns		F	1
D 0290		✓	✓		+ d	✓			✓				(✓)	(✓)	(✓)		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓		+ ✓	+ ✓	+ ✓	✓	✓	✓	✓		M	3
D 0292		(✓)	✓	+		+ d ✓	✓	✓	ns	ns		ns					+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)	+ ✓	+ ✓		+ ✓	✓	(✓)			F	2
D 0293	✓	✓	✓	✓	✓	✓			✓				✓		✓	✓	✓	✓	+ ✓	✓	✓	✓	✓	+ ✓	✓	✓	+ ✓	+ ✓	✓		+ ✓	✓		ns		F	2
D 0294	+		✓	✓	✓													✓		✓	✓	✓	✓				✓	✓								M	3
D 0295		+ ✓		✓		+								ns		(✓)		✓			+ ✓		✓	✓			✓	+ ✓		ns	✓					M	3
D 0298	+ d	(✓)	✓	✓	✓	✓		(✓)		(✓)	ns	ns	ns				+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓		✓	✓	+ ✓	+ (✓)					T ✓	M	2

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
D 5225		✓	✓	+	(✓)	+			✓	✓	✓	✓	ns				+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)	✓	+ ✓	✓	✓	+ ✓		ns	(✓)		+ ✓	✓		M	4
D 5240	✓	✓	+	✓	+ (✓)	+ d ✓	(✓)			✓			✓	ns			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	(✓)	✓	+ (✓)							RMM (✓)	F	1
D 5241	+	✓	✓																											ns		ns				M	1
D 5243	✓								ns						ns							+ ✓	+ ✓	✓				✓		ns		(✓)		ns		M	5
D 5245						✓													+ ✓	+ ✓	+ ✓			+ ✓				✓		ns	✓			ns		M	3
D 5246			+			+									ns		+ (✓)	+ ✓	+ (✓)	+ (✓)	+ ✓	+ (✓)						(✓)		ns					F	1
D 5247	✓	+ d ✓	+ ✓	✓	+ ✓	✓		ns		✓					✓	(✓)	+ ✓	+ ✓	+ ✓	+ (✓)	+ ✓	+ ✓	✓	✓	+ ✓		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓			✓		M	1
D 5276	✓	(✓)		+ ✓	✓	+ ✓				ns			ns				+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓			+ ✓	+ (✓)	+ ✓	+	(✓)	ns				M	4
G 1092			✓	✓	✓	+ ✓	(✓)	✓		ns		✓				✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)				F	3
G 1093	✓	✓	✓	+	✓	+ d	(✓)		ns	ns		✓		✓			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	✓		+ ✓	+ ✓	+ ✓	+ ✓	✓	(✓)	✓	✓	D ✓	M	3
G 1097					✓							ns												ns								ns				F	8
G 1098	+ ✓	✓	✓		+ ✓	✓																			(✓)											M	1
G 1099	+ d	✓	✓	✓	✓	✓	(✓)	(✓)	ns	ns				(✓)	(✓)	(✓)	+ ✓	✓	+ ✓	✓	+ ✓	✓	✓	+ ✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	(✓)			M	3
G 1100	+		✓						ns	ns																						ns		ns		F	4
D 0296					✓	✓	(✓)		ns	ns	ns		ns								+ ✓		✓	✓	✓		✓	✓		ns	✓	ns				M	2
D 5229	+ d ✓	ns	✓	✓	✓	✓	✓	✓		(✓)		✓	✓			✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	(✓)	+ ✓	+ ✓	✓	✓				F	6
D 5230	+ d ✓	✓	✓	+ ✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	ns	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	+ ✓	✓	+ ✓	+ ✓	✓	✓	+ ✓	ns	✓	✓	2MM ✓	F	8
D 5237	+ ✓				✓	+ d ✓											+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	+ ✓		+ ✓	+ (✓)	(✓)	+ (✓)	✓		✓			F	1
D 5238	+ d ✓	ns	ns	✓		✓	(✓)							(✓)	✓	ns		+ ✓			+ ✓		✓	✓			+ ✓	+ ✓		+ ✓	+ ✓	✓				M	4
D 5228	✓											ns	✓		✓		(✓)	✓					+ ✓	ns	✓		✓	+ ✓			✓		✓			F	7
H 3122		+ d ✓		+ ✓							ns			✓			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓		(✓)	(✓)	(✓)	+ ✓	+ ✓					F	3
H 3126		ns	(✓)										ns		ns						✓			✓												F	0.5
H 3129		✓	+ (✓)		+ ✓			(✓)				ns						+ ✓	+ (✓)	✓	+ ✓	✓	(✓)		+ ✓	(✓)		+ ✓							F	2

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
H 3132	ns			ns		ns			ns	ns					ns	ns			✓		(✓)			✓												M	0.5
H 3136				✓							ns			(✓)		ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓		✓	✓			✓	+ ✓		+ ✓						F	3
H 3137	+ ✓	✓	(✓)						✓	(✓)			(✓)		✓	(✓)	+ ✓	✓	+ (✓)	+ ✓	+ ✓	+ (✓)	✓	✓	+ ✓		✓	(✓)	✓	✓	+ ✓					M	8
H 3141	+ ✓	+ ✓	ns	✓	✓				✓			(✓)		(✓)		ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓		+ ✓	+ (✓)	+ ✓	+ ✓	✓					M	2
H 3142	+ ✓	+ ✓	✓										(✓)				+ (✓)	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	✓		(✓)	✓	+ ✓	+ ✓	+ ✓					M	4
H 3151		ns	✓	ns	(✓)	+ d ✓			ns	ns	ns	ns	ns	ns	ns	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓	(✓)	(✓)	+ ✓			+ (✓)					F	3
H 3176	+ ✓		✓													(✓)		+ ✓			+ ✓						✓	(✓)								M	1
H 3179	+ ✓		+ ✓				✓						✓			✓		✓					✓		✓		+ ✓	+ (✓)			+ ✓	(✓)				F	9
H 3181	+ d ✓	(✓)	✓															✓	+ (✓)				(✓)	✓	✓		✓									M	8
H 3194	+ d ✓		+ ✓	✓		(✓)			ns	ns	ns	ns	ns	+ ✓	ns	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓	+ ✓	✓	(✓)		+ (✓)					M	4
H 3195			+ ✓	(✓)	+ d ✓	✓			ns	ns	ns	ns	ns	ns			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓			✓	+ ✓	+ ✓	+ ✓	✓	(✓)				F	2
H 3196	+ ✓		✓														+ ✓	+ ✓	+ ✓		+ ✓		✓	✓	(✓)	(✓)	✓									M	5
G 3310	+ ✓		✓			✓				✓						ns	+ ✓	+ ✓			+ ✓	✓	+ ✓	(✓)			✓	+ (✓)		+ ✓						M	1
G 3311	+ ✓	+ ✓	✓	(✓)		(✓)	(✓)									✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓		(✓)	(✓)		✓	+ ✓		+ ✓	(✓)		✓			F	4
G 3334			(✓)						ns	ns	ns	ns	ns	ns			✓	+ ✓		(✓)	+ ✓		+ ✓	+ ✓		✓	✓	+ ✓								F	6
H 3917	+ d		+ ✓	✓	✓	✓			✓	✓		✓		ns	✓		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	+ ✓	+ ✓	✓	+ ✓	+ ✓	✓				M	4
H 3918							✓						+	+ ✓			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓		✓	+ ✓		+ (✓)	✓					F	1
D 5655					+	✓					✓					(✓)		+ ✓	+ ✓	+ ✓	+ ✓		✓	✓			✓	+ ✓	+ ✓		+ ✓					M	1
B 6251	✓		+		+ (✓)																						ns	+ ✓			✓	(✓)				M	1
B 6257	+	+ ✓		✓							ns					ns																				M	7
B 6261			+ ✓	✓	+ ✓	✓			+	ns	✓		✓	✓	✓	✓		✓	✓		✓	✓					✓	+ ✓			✓	✓				M	2
G 3552					+													+ ✓		+ ✓	+ ✓	+ ✓	✓	(✓)											M	2
G 3553																			(✓)						+ ✓										F	0.8

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
B 3539	✓	+ ✓	+ ✓	+ ✓	+ ✓	+	✓		✓	+ ✓	+ ✓	✓	(✓)	+ ✓	+ ✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓			I C ✓ +	M	2
G 0249			✓	+ ✓		+ d	✓					(✓)					+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓		✓								M	4
K 0937		+		+ ✓		✓					ns		✓				✓	+ ✓	+ (✓)	+ ✓	+ ✓		+ ✓	+ ✓	+ ✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)			C + ✓	M	1
K 0952		+		+ ✓					ns						✓			✓			(✓)		✓	✓			✓	+ ✓			+ ✓	(✓)				F	3
K 0973		✓		✓																					✓											F	1
K 0985	+		✓					ns																												M	2
K 0995	+ (✓)		+ ✓	+ ✓	+ ✓		(✓)	ns				ns					✓						✓		✓		✓	+ ✓	+ ✓		✓					F	5
G 2935		+		+ ✓	✓	+ d ✓											+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ (✓)	+ ✓	✓		(✓)	(✓)	+ ✓				✓		M	3
G 2936			✓		+																							✓			✓					M	3
K 1327			+ ✓		+ d	✓	ns	(✓)		ns						ns	✓	+ ✓	+ ✓		+ ✓	+ ✓	✓	✓				+ (✓)	+ ✓	+ ✓	+ ✓					M	3
K 1471		(✓)			✓		✓	✓	✓	✓			✓	+ ✓	✓	✓	+	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓			✓					M	1
H 4248	ns	ns	ns	ns	+	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns									ns	ns	ns	ns	ns	ns	+ ✓	ns	ns	ns		M	1
K 1230	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		+ ✓		+ ✓	+ ✓		✓		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		M	1
R 1647	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ d ✓	✓		(✓)		✓	✓	✓	(✓)	✓		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓		(✓)	✓	+ ✓	(✓)		F	0.5
R 1678		✓	+ ✓	+ ✓	✓	+ d ✓		(✓)		✓							+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)	✓	(✓)		+ (✓)								M	3
R 1744			+ ✓	✓	✓	+																														M	4
R 1746	+ ✓	+ ✓		+ ✓	✓	✓	(✓)	(✓)	✓	ns																										M	2
R 1758	+ ✓	+ ✓	✓	+ ✓	+ (✓)	(✓)							(✓)			+ ✓	✓	+ ✓			✓		+ ✓	ns			+ ✓	(✓)				(✓)				M	4
R 1895													ns					+ ✓					+ ✓												F	0.5
A 2865	✓										ns					ns										ns	ns								M	M
7662		✓	+ ✓	✓	✓	✓			✓	✓	+ ✓	+ ✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)	+ ✓	✓	+ ✓	+ ✓	✓	✓	✓	+ (✓)		(✓)		(✓)		(✓)		M	M
7663	ns		+ ✓	ns	(✓)								ns	ns	ns	ns	✓	✓			(✓)		✓	✓			✓	(✓)								M	M
D 3694	+ d ✓	ns	✓	ns	✓	ns		ns	ns	ns	✓	ns	ns	ns	ns	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ (✓)	+ ✓	✓	✓	ns	✓	✓	✓	ns	✓	ns	✓	ns	LMM (✓)	F	2

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
G 19146	+ d ✓	+ d ✓	+ ✓	+ ✓	+ ✓	+ ✓			+ ✓	ns	+ ✓	+ ✓		ns	(✓)	✓	+ (✓)	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	(✓)		✓	+ ✓	(✓)			(✓)					M	2
D 17725		✓																																		F	2
G 18477						+																														F	2
P119 -413		+	✓	✓	+ ✓	✓					ns			ns																						M	2
D 2191				+ d ✓													(✓)	(✓)	(✓)	(✓)	(✓)	(✓)	(✓)													M	4
D 2285		✓	+ ✓	+ (✓)		+ d ✓											✓	+ ✓		+ ✓	+ ✓	(✓)	✓	(✓)	✓		ns	(✓)								M	2
D 2334				✓		✓	(✓)							ns					+ (✓)		+ ✓							+ (✓)								M	4
D 2827	+ d ✓	✓	+ ✓	+ ✓	✓	+						✓	✓	✓			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓		+ ✓	✓	+ ✓	+ (✓)	✓	✓			M	4
P119 -412	+ ✓	+	+ ✓	+ ✓	✓	+ ✓	✓	✓	+ ✓	+ ✓	+ ✓	+	✓	✓	+ ✓	✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	✓	✓	✓	+ ✓	✓	✓	✓		F	2
P 18943										ns								+ ✓					✓													F	2
D 17727														✓			+ ✓	+ ✓	(✓)	(✓)		(✓)	+ ✓	+ ✓			✓	✓			✓	(✓)				M	2
G 18406	✓	+ ✓	✓	✓	+ ✓	✓	✓	✓	+ d ✓	+ ✓	+ d ✓	✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ (✓)	+ ✓	+ ✓	+ ✓	✓	(✓)	✓	✓		M	4
G 18408	+																	+ ✓		+ (✓)	✓		✓	(✓)												F	1
G 18461			(✓)	(✓)	✓													+ ✓	+ (✓)													✓				M	2
G 18470	✓	✓	✓	✓	✓	+				✓			✓	✓		(✓)	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	+ ✓	✓	✓	+ ✓	✓	+ ✓	✓	(✓)	+ ✓	+ ✓		F	2
G 18473				(✓)		+											+ (✓)						✓		+ ✓											F	2
G 18482	+ d ✓		✓																								(✓)									F	2
G 18483						+																						(✓)								M	1
G 18484	+ (✓)		✓	(✓)											ns												(✓)									M	2
G 18487			✓		+ d ✓							ns																								M	1
G 19112	✓		+ ✓															+ ✓	+ ✓		+ ✓		+ ✓	✓												F	4
G 19136		+ ✓			+										ns								ns													F	1
D 22287	+ ✓	+ ✓	✓	+	✓	✓											+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ (✓)	✓	✓	✓	+ ✓	+ ✓	+ ✓		(✓)	✓	✓		M	M

Appendix VIII. (Page 6 of 6) – Page 184

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
103				+ ✓		+ ✓			ns	ns									(✓)		+ ✓			✓	+ ✓			+ (✓)								M	2
200		ns	+ ✓		+ ✓				ns	ns																										M	2
Total Gross	38	24	28	24	27	26	0	0	4	3	5	4	1	5	5	3	52	67	60	52	68	46	29	22	28	2	26	51	25	32	21	0	3	1	Total Males		73
% Gross	32	21	24	21	23	22	0	0	3	3	4	3	1	4	4	3	44	57	51	44	58	39	25	19	24	2	22	44	21	27	18	0	3	1	Total Females		44
Total Lesns	57/113	51/109	70/113	59/111	61/116	61/113	25/114	17/111	18/99	17/94	11/101	16/100	26/103	24/103	17/102	23/102	63	80	66	62	80	57	77/116	70/111	60/115	27/113	67/111	75/115	35/113	36/99	52/116	28/104	16/115	14/107			
% Lesns	50	47	62	53	53	54	22	15	18	18	11	16	25	23	17	23	54	68	56	53	68	49	66	63	52	24	60	65	31	36	45	27	14	13			
Other sites	Duodenum Total Lesns 1/112 (1%)				Ileum Total Lesns 1/115 (1%)				Colon Total Gross 2 (2%)				Colon Total Lesns 2/113 (2%)				L mammary Total Lesns 2/42 (5%)				R mammary Total Lesns 3/38 (8%)				Thymus Total Lesns 1/99 (1%)										

All sites were examined in 117 possums for the presence of macroscopic lesions.
Total (%) Gross is the total number (%) of macroscopic lesions present at each of the sites examined.
The numerator for Total Lesns is the total number of macroscopic plus microscopic lesions at each of the sites examined.
The denominator for Total Lesns represents the total number of observations where tissues/organs from less than 117 animals were sampled for histopathological examination.
Of the 117 tuberculous possums, the diagnosis was confirmed as follows:

88 had *M. bovis* cultured from at least one macroscopic lesion
21 with macroscopic lesions were confirmed by histopathological examination only
8 had no visible macroscopic lesions and were confirmed by histopathological examination only

Appendix IX. Details of distribution of macroscopic and microscopic lesions at five general body sites in 117 tuberculous possums derived from field studies (Page 1 of 2) – Page 185

	Superficial		Head and Neck		Respiratory tract		Gastrointestinal tract		Other sites	
Tag No.	Gross	G & M	Gross	G & M	Gross	G & M	Gross	G & M	Gross	G & M
B6101	+	✓		✓	+	✓	+	✓	+	✓
B6102				✓	+	✓	+	✓		
B6103	+	✓			+	✓		✓	+	✓
B6104								✓		
B6106	+	✓			+	✓				
B6110	+	✓		✓	+	✓	+	✓		✓
B6111	+	✓		✓	+	✓		✓	+	✓
B6113		✓								
B6114	+	✓								
B6115		✓		✓	+	✓	+	✓		✓
B6117		✓	+	✓	+	✓	+	✓		✓
B6118	+	✓			+	✓	+	✓	+	✓
B6119		✓	+	✓	+	✓	+	✓	+	✓
B6122		✓			+	✓				
B6123	+	✓		✓	+	✓	+	✓		
D0218	+	✓		✓	+	✓	+	✓	+	✓
D0280	+	✓				✓		✓		✓
D0290	+	✓		✓	+	✓	+	✓	+	✓
D0292	+	✓		✓	+	✓	+	✓	+	✓
D0293		✓		✓	+	✓	+	✓	+	✓
D0294	+	✓				✓		✓		
D0295	+	✓		✓	+	✓	+	✓		✓
D0298	+	✓		✓	+	✓		✓	+	✓
D5225	+	✓		✓	+	✓	+	✓	+	✓
D5240	+	✓		✓	+	✓	+	✓		✓
D5241	+	✓								
D5243		✓			+	✓		✓		✓
D5245		✓			+	✓		✓		✓
D5246	+	✓			+	✓		✓		
D5247	+	✓		✓	+	✓	+	✓	+	✓
D5276	+	✓			+	✓	+	✓	+	✓
G1092	+	✓		✓	+	✓	+	✓	+	✓
G1093	+	✓		✓	+	✓	+	✓	+	✓
G1097		✓								
G1098	+	✓						✓		
G1099	+	✓		✓	+	✓	+	✓	+	✓
G1100	+	✓								
D0296		✓		✓	+	✓		✓		✓
D5229	+	✓		✓	+	✓	+	✓	+	✓
D5230	+	✓		✓	+	✓	+	✓	+	✓
D5237	+	✓			+	✓	+	✓	+	✓
D5238	+	✓		✓	+	✓	+	✓	+	✓
D5228		✓		✓	+	✓	+	✓		✓
H3122	+	✓		✓	+	✓	+	✓	+	✓
H3126		✓				✓				
H3129	+	✓		✓	+	✓	+	✓		
H3132						✓				
H3136		✓		✓	+	✓	+	✓	+	✓
H3137	+	✓		✓	+	✓	+	✓	+	✓
H3141	+	✓		✓	+	✓	+	✓	+	✓
H3142	+	✓		✓	+	✓		✓	+	✓
H3151	+	✓			+	✓	+	✓	+	✓
H3176	+	✓		✓	+	✓		✓		
H3179	+	✓		✓		✓	+	✓	+	✓
H3181	+	✓			+	✓		✓		
H3194	+	✓	+	✓	+	✓	+	✓	+	✓
H3195	+	✓			+	✓	+	✓	+	✓
H3196	+	✓			+	✓		✓		
G3310	+	✓		✓	+	✓	+	✓	+	✓
G3311	+	✓		✓	+	✓	+	✓	+	✓
G3334		✓			+	✓	+	✓		
H3917	+	✓		✓	+	✓	+	✓	+	✓
H3918			+	✓	+	✓	+	✓	+	✓

	Superficial		Head and Neck		Respiratory tract		Gastrointestinal tract		Other sites	
Tag No.	Gross	G & M	Gross	G & M	Gross	G & M	Gross	G & M	Gross	G & M
D5655	+	✓		✓	+	✓	+	✓	+	✓
B6251	+	✓					+	✓		✓
B6257	+	✓								
B6261	+	✓	+	✓		✓	+	✓		✓
G3552	+	✓			+	✓				
G3553						✓	+	✓		
B3539	+	✓	+	✓	+	✓	+	✓	+	✓
G0249	+	✓		✓	+	✓		✓		
K0937	+	✓		✓	+	✓	+	✓	+	✓
K0952	+	✓		✓		✓	+	✓	+	✓
K0973		✓						✓		
K0985	+	✓								
K0995	+	✓		✓		✓	+	✓	+	✓
G2935	+	✓			+	✓		✓	+	✓
G2936	+	✓						✓		✓
K1327	+	✓		✓	+	✓	+	✓	+	✓
K1471		✓	+	✓	+	✓	+	✓		✓
H4248	+	✓						ns	+	✓
K1230		ns		ns	+	✓		ns		ns
R1647	+	✓		✓	+	✓	+	✓	+	✓
R1678	+	✓		✓	+	✓	+	✓		
R1744	+	✓								
R1746	+	✓		✓						
R1758	+	✓	+	✓	+	✓	+	✓		✓
R1895					+	✓				
A42865		✓								
47662	+	✓	+	✓	+	✓	+	✓		✓
47663	+	✓				✓		✓		
D3694	+	✓		✓	+	✓		✓		✓
G39146	+	✓	+	✓	+	✓	+	✓		✓
D47725		✓								
G38477	+	✓								
P119413	+	✓								
D2191	+	✓				✓				
D2285	+	✓			+	✓		✓		
D2334		✓		✓	+	✓	+	✓		
D2827	+	✓		✓	+	✓	+	✓	+	✓
P119412	+	✓	+	✓	+	✓	+	✓	+	✓
P28943					+	✓				
D47727				✓	+	✓		✓		✓
G38406	+	✓	+	✓	+	✓	+	✓	+	✓
G38408	+	✓			+	✓				
G38461		✓			+	✓				✓
G38470	+	✓		✓	+	✓	+	✓	+	✓
G38473	+	✓			+	✓	+	✓		
G38482	+	✓						✓		
G38483	+	✓						✓		
G38484	+	✓						✓		
G38487	+	✓								
G39112	+	✓			+	✓				
G39136	+	✓								
D2287	+	✓			+	✓	+	✓	+	✓
103	+	✓			+	✓	+	✓		
200	+	✓								
TOTAL	88	108/116	12	60/116	81	92	62	88/115	47	67/116
%	75	93	10	52	69	79	53	77	40	58

Superficial = left and right inguinal, left and right deep and superficial axillary lymph nodes
Head and Neck = left and right palatine tonsils; left and right mandibular, parotid, and deep and superficial cervical lymph nodes
Respiratory tract = left and right cranial and caudal, and right middle and accessory lung lobes; left and right anterior mediastinal lymph nodes
Gastrointestinal tract = mesenteric, gastric, and hepatic lymph nodes; liver, duodenum, ileum, colon
Other sites = left and right kidney, adrenal and mammary glands; spleen, bone marrow, thymus
Gross: all sites were examined in 117 possums for the presence of macroscopic lesions
G & M: the total number of macroscopic (gross) plus microscopic lesions at each of the sites examined
Total G & M: the denominator represents the total number of observations where tissues/organs from less than 117 animals were sampled for histopathological examination

Appendix X. Details of distribution of macroscopic and microscopic lesions in 20 terminally ill possums derived from field studies

Key

No.	tag number
LSA	left superficial axillary lymph node
RSA	right superficial axillary lymph node
LDA	left deep axillary lymph node
RDA	right deep axillary lymph node
LI	left inguinal lymph node
RI	right inguinal lymph node
LT	left palatine tonsil
RT	right palatine tonsil
LM	left mandibular lymph node
RM	right mandibular lymph node
LP	left parotid lymph node
RP	right parotid lymph node
LR	left (retropharyngeal) deep cervical lymph node
RR	right (retropharyngeal) deep cervical lymph node
LCC	left (caudal) superficial cervical lymph node
RCC	right (caudal) superficial cervical lymph node
LCR	left cranial lung lobe
LCD	left caudal lung lobe
RCR	right cranial lung lobe
RMD	right middle lung lobe
RCD	right caudal lung lobe
RAC	right accessory lung lobe
LB	left (bronchial) anterior mediastinal lymph node
RB	right (bronchial) anterior mediastinal lymph node
M	mesenteric lymph node
G	gastric lymph node
H	hepatic lymph node
L	liver
LK	left kidney
RK	right kidney
S	spleen
BM	bone marrow
LA	left adrenal gland
RA	right adrenal gland
Other	D = duodenum
	I = ileum
	C = colon
	LMM = left mammary gland
RMM = right mammary gland	
	2MM = left and right mammary glands
	T = thymus
Sex	F = female, M = male
Age	in years, or:
	I = immature
	M = mature
+	macroscopic lesion confirmed by histopathology and/or culture
d	discharging sinus
✓	microscopic lesion with acid fast organisms (AFOs)
(✓)	microscopic lesion characteristic of tuberculosis without AFOs
ns	not sampled

Appendix X. Details of distribution of macroscopic and microscopic lesions in 20 terminally ill possums derived from field studies (Page 1 of 2) – Page 188

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Other	Sex	Age
R 1883	✓	+ d ✓	✓	+	✓	+ d ✓	✓	✓	✓	✓	✓	✓	ns	(✓)	✓	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	✓	I ✓	M	3
River -dale	✓	ns	+ d	+ d	ns	✓	✓	✓	ns	ns	✓	ns	✓	✓			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	+ ✓	+ ✓	✓	✓	+ ✓	+ ✓	C + ✓ RMM + d ✓ LMM ✓	F	M
D 2950	+ d ✓	+ d ✓	ns	ns	ns	ns	✓	✓	+	+	ns	ns	+ ✓	+ ✓	ns	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+	+	ns	✓	ns	ns	+ ✓	(✓)	ns	ns		M	3
D 2906	✓	✓	ns	ns	✓	✓	✓		ns	✓	+ ✓	ns	ns	ns	+ ✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓	ns	+	+ ✓	+ ✓	ns	+ ✓	ns		ns		F	0.6
D 2219	ns	ns	✓	✓	✓	+ d ✓			✓	ns		✓	✓	✓	✓		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓		✓	+	+ ✓		✓			ns	ns		F	M
W 005	+ d		✓	✓			✓	✓	ns	ns	+ d	ns	ns	ns	+ ✓		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+	+ ✓	+ ✓	+ ✓	ns	ns	✓	✓	D C ✓	M	3
D 2917	+ d			+ ✓	✓	(✓)					ns	+ d	ns				+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ (✓)	+ ✓	ns	✓	+ ✓			ns	ns		+ ✓		M	3
D 2143	+ ✓		+ d ✓	✓	✓			(✓)					✓	✓			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓	✓	✓	(✓)	✓	✓		M	M
D 2319	ns	ns	ns	ns	✓	✓		ns	✓	✓	✓	✓	✓	+ ✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	+	+ ✓	+ ✓	+ ✓	+ ✓	+	+		✓		C + D I ✓	M	M
D 3581	✓	ns	ns	ns	✓	✓	✓		ns	ns	ns	✓	✓	✓	✓		+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓	✓	+	✓	+ ✓		✓	✓			D + ✓	M	M
D 2841	ns	ns	+	ns	+ d	ns		✓	ns	ns	ns	Ns	ns	ns	ns	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	ns	✓	+ ✓	✓	C + ✓ I ✓	M	M
D 2801	✓	✓	✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+	+ ✓	+ ✓	✓	✓	✓	✓	✓	✓	✓	(✓)	✓	✓	RMM ✓	F	4
P 10846	✓	✓	+ ✓	✓	✓	+ d ✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓	+ ✓	✓	✓	✓	(✓)	✓	✓		M	M
D 47792	✓	✓	✓	✓	+ ✓	+			✓	✓	✓	✓	✓	✓	✓	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+	+ ✓	✓	+ ✓	ns	ns	✓	+ ✓	+ ✓	✓	(✓)	✓	+ ✓	C + ✓ 2MM ✓	F	6
H 5718	+ ✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓	✓	+ ✓	+ ✓	✓	✓	ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	✓	+ ✓	+ ✓	+ ✓	C + ✓ D I ✓ 2MM ✓	F	I
D 3624	+ ✓	+	+ ✓	+ ✓	ns	ns			+ ✓		+ ✓	+ ✓		ns			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	✓	✓	(✓)	✓	✓		+ ✓	✓	(✓)				M	2
D 2401		ns			✓	ns			ns	ns			✓	✓		✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	✓	✓	✓			✓		✓		D ✓	F	M
Erin-dale	✓		+	ns	✓	+ d ✓	✓		✓	ns	ns	✓	ns			✓	✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	✓	+ ✓	✓	✓	✓		+ ✓	✓	(✓)		✓		M	7
D 2842	+										ns	ns	ns	ns			+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	✓	+ ✓	ns	ns	ns	+ ✓	+ ✓	+ ✓	✓	ns	ns	ns	D I ✓	M	M
W 002	ns	ns	ns	ns	ns	ns				ns	+ d	ns	✓	✓		ns	+ ✓	+ ✓	+ ✓	+ ✓	+ ✓	+ (✓)	✓	+ ✓	ns	ns	ns	+ ✓	ns	(✓)	ns	ns	ns	ns	ns	F	M

Appendix X. (Page 2 of 2) – Page 189

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	LA	RA	Sex	
Total Gross	7	3	7	5	3	6	0	0	2	1	4	3	2	2	2	0	19	20	20	20	20	19	12	12	13	4	7	10	9	10	4	1	4	4	Total Males	12
% Gross	35	15	35	25	15	30	0	0	10	5	20	15	10	10	10	0	95	100	100	100	100	95	60	60	65	20	35	50	45	50	20	5	20	20	Total Females	8
Total Lesns	15/16	8/13	13/15	12/13	14/16	12/15	9	8/19	10/14	8/12	11/14	11/13	12/13	13/15	10/18	6/14	20	20	20	20	20	20	20	20	17/18	15/15	16/16	20	13/18	15/18	15/16	12/15	11/16	11/15		
% Lesns	94	61	87	92	88	80	45	42	71	67	79	85	92	87	56	43	100	100	100	100	100	100	100	100	94	100	100	100	72	83	94	80	69	73		
Other sites	Duodenum Total Gross		1	(5%)	Duodenum Total Lesns		6/16	(38%)	Ileum Total Lesns		5/16	(31%)	Colon Total Gross		5	(25%)	Colon Total Lesns		6/16	(38%)	L mammary Total Lesns		3/6	(50%)	R mammary Total Gross		1	(13%)	R mammary Total Lesns		4/6	(67%)				

All sites were examined in 20 possums for the presence of macroscopic lesions.
Total (%) Gross is the total number (%) of macroscopic lesions present at each of the sites examined.
The numerator for Total Lesns is the total number of macroscopic (gross) plus microscopic lesions at each of the sites examined.
The denominator for Total Lesns represents the total number of observations where tissues/organs from less than 20 animals were sampled for histopathological examination.

Appendix XI. Details of distribution of macroscopic and microscopic lesions at five general body sites in 20 terminally ill possums derived from field studies

	Superficial		Head and Neck		Respiratory tract		Gastrointestinal tract		Other sites	
Tag No.	Gross	G & M	Gross	G & M	Gross	G & M	Gross	G & M	Gross	G & M
R1883	+	✓		✓	+	✓	+	✓	+	✓
Riverdale	+	✓		✓	+	✓	+	✓	+	✓
D2950	+	✓	+	✓	+	✓	+	✓	+	✓
D2906		✓	+	✓	+	✓	+	✓	+	✓
D2219	+	✓		✓	+	✓	+	✓		✓
W005	+	✓	+	✓	+	✓	+	✓	+	✓
D2917	+	✓	+	✓	+	✓	+	✓	+	✓
D2143	+	✓		✓	+	✓	+	✓		✓
D2319		✓	+	✓	+	✓	+	✓	+	✓
D3581		✓		✓	+	✓	+	✓	+	✓
D2841	+	✓		✓	+	✓	+	✓	+	✓
D2801		✓		✓	+	✓		✓		✓
P10846	+	✓		✓	+	✓	+	✓		✓
D47792	+	✓		✓	+	✓	+	✓	+	✓
H5718	+	✓	+	✓	+	✓	+	✓	+	✓
D3624	+	✓	+	✓	+	✓		✓	+	✓
D2401		✓		✓	+	✓		✓		✓
Erindale	+	✓		✓	+	✓	+	✓	+	✓
D2842	+	✓			+	✓	+	✓	+	✓
W002		ns	+	✓	+	✓	+	✓		✓
TOTAL	14	19/19	8	19	20	20	17	20	14	20
%	70	100	40	95	100	100	85	100	70	100

Superficial = left and right inguinal, left and right deep and superficial axillary lymph nodes
Head and Neck = left and right palatine tonsils; left and right mandibular, parotid, and deep and superficial cervical lymph nodes
Respiratory tract = left and right cranial and caudal, and right middle and accessory lung lobes; left and right anterior mediastinal lymph nodes
Gastrointestinal tract = mesenteric, gastric, and hepatic lymph nodes; liver, duodenum, ileum, colon
Other sites = left and right kidney, adrenal and mammary glands; spleen, bone marrow, thymus
Gross: all sites were examined in 20 possums for the presence of macroscopic lesions
G & M: the total number of macroscopic (gross) plus microscopic lesions at each of the sites examined
Total G & M: the denominator represents the total number of observations where tissues/organs from less than 20 animals were sampled for histopathological examination
ns = not sampled

Appendix XII. Details of distribution of microscopic lesions in 38 possums infected via the I/D route into the neck with BCG

Key

No.	possum number
LSA	left superficial axillary lymph node
RSA	right superficial axillary lymph node
LDA	left deep axillary lymph node
RDA	right deep axillary lymph node
LI	left inguinal lymph node
RI	right inguinal lymph node
LT	left palatine tonsil
RT	right palatine tonsil
LM	left mandibular lymph node
RM	right mandibular lymph node
LP	left parotid lymph node
RP	right parotid lymph node
LR	left (retropharyngeal) deep cervical lymph node
RR	right (retropharyngeal) deep cervical lymph node
LCC	left (caudal) superficial cervical lymph node
RCC	right (caudal) superficial cervical lymph node
LCR	left cranial lung lobe
LCD	left caudal lung lobe
RCR	right cranial lung lobe
RMD	right middle lung lobe
RCD	right caudal lung lobe
RAC	right accessory lung lobe
LB	left (bronchial) anterior mediastinal lymph node
RB	right (bronchial) anterior mediastinal lymph node
M	mesenteric lymph node
G	gastric lymph node
H	hepatic lymph node
L	liver
LK	left kidney
RK	right kidney
S	spleen
BM	bone marrow
Sex	F = female, M = male
Age	I = immature
	A = adult
Lesns	lesions
✓	microscopic lesion with acid fast organisms (AFOs)
(✓)	microscopic lesion characteristic of tuberculosis without AFOs
ns	not sampled

Appendix XII. Details of distribution of microscopic lesions in 38 possums infected via the I/D route into the neck with BCG – Page 192

No.	LSA	RSA	LDA	RDA	LI	RI	LT	RT	LM	RM	LP	RP	LR	RR	LCC	RCC	LCR	LCD	RCR	RMD	RCD	RAC	LB	RB	M	G	H	L	LK	RK	S	BM	Skin		Sex	Age
1																																	✓		M	A
2				✓								ns			ns																		✓		F	A
3			✓																														✓		M	A
5																																	✓	W	M	A
23										ns	ns	ns																					✓	E	F	A
24									ns															ns									✓	E	M	A
9																																	✓	K	F	A
10				✓																													✓		F	A
11				✓						ns													ns										✓		F	A
12	✓			✓												ns																	✓	1	M	A
13				✓												ns																	✓		F	A
14																							ns	ns									✓		M	A
15				✓																				ns									✓		F	A
4																ns									(✓)		(✓)	(✓)			(✓)		✓		F	A
6				(✓)								(✓)																					✓		M	A
7		(✓)	✓	(✓)		✓																	✓		✓	✓	(✓)	(✓)			(✓)		✓		M	A
8										ns															(✓)								✓	W	F	A
9A			(✓)	✓							(✓)					✓							(✓)										✓	E	M	A
10A			✓	(✓)																								(✓)					✓	E	M	A
16			✓	✓																													✓	K	F	A
17			✓	✓																													✓		M	1
18			(✓)	✓																			ns										✓	2	M	1
19				✓																				ns									✓		F	A
20		(✓)	✓	✓							ns																✓	(✓)					✓		F	A
21			✓	(✓)		(✓)																					✓	(✓)			(✓)		✓		M	A
11A	✓	(✓)												(✓)			(✓)							✓	(✓)	(✓)	✓	(✓)			✓		(✓)	W	M	I
12A	(✓)	(✓)	(✓)	(✓)		(✓)							(✓)	(✓)	ns	(✓)			(✓)					(✓)		(✓)	✓	(✓)			(✓)		✓	E	F	A
13A			(✓)					(✓)	(✓)				✓												✓		✓						✓	E	M	A
14A	(✓)		(✓)	✓	(✓)	(✓)					(✓)		✓	(✓)									(✓)	(✓)	(✓)	✓	(✓)	(✓)			(✓)	(✓)	✓	K	M	A
16A			✓						ns																		(✓)						(✓)		F	A
25	(✓)			(✓)								ns													(✓)		(✓)						(✓)	3	M	A
15A			(✓)													ns																	(✓)		M	A
17A		✓	✓	✓								✓				(✓)							✓	✓			✓				✓		(✓)	W	M	A
18A				(✓)					✓																		(✓)						(✓)	E	M	A
19A		(✓)	(✓)	(✓)								ns													(✓)		(✓)						✓	E	M	A
20A		(✓)	✓	(✓)							(✓)	ns	(✓)										(✓)		✓		(✓)	(✓)					✓	K	F	I
21A				(✓)							(✓)	(✓)		(✓)	(✓)									(✓)			(✓)				(✓)		(✓)	4	F	A
22																																	✓		M	A
Total Lesns	5	7	17	24	1	4	0	1	2/36	0/35	4/36	3/33	4	4	1/36	3/34	1	0	1	0	0	0	5/35	5/34	9	4	15	9	0	0	8	1	38		22 Males	
% Lesns	13	18	45	63	3	11	0	3	6	0	11	9	11	11	3	9	3	0	3	0	0	0	14	15	24	11	39	24	0	0	21	3	100		16 Females	

The numerator for Total Lesns is the total number of microscopic lesions at each of the sites examined.
The denominator for Total Lesns represents the total number of observations where tissues/organs from less than 38 animals were sampled for histopathological examination.

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ERRATA

Page Number	Error	Amendment
v	(Landcare Research, **Linclon**)	Linc**o**ln
46	…cleared to the **orophranynx** and swallowed.	oroph**a**rynx
51	…samples were **randomly** selected for histology.	**arbitrarily**
72	…in which no **macrosocpic** lesions were observed.	macros**c**opic
82	…possums with advanced **lesions** in this study…	**disease**
100	…of the macrophage **system probably** takes…	system **which** probably
121	…without **discernable** pulmonary parenchymatous foci.	discernible
129	…of binding **in** both lung and peripheral lymph nodes…	**with**
132	…a possum sleeping on three **carcasses** in one den.	carcases