

## Experimental infection of badgers (*Meles meles*) with *Mycobacterium bovis*

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(Accepted 26 September 1986)

### SUMMARY

The intradermal inoculation of four badgers with small numbers of *Mycobacterium bovis* resulted in localized lesions with ulceration which slowly healed by 5 months after inoculation. Lesions of generalized tuberculosis were seen in three badgers, one of which died at 17 months post-inoculation and in the remaining two killed 22 months post-inoculation. In the fourth badger lesions were confined to the draining lymph node of the inoculation site but *M. bovis* was isolated from the liver. Monthly clinical sampling of faeces, urine, tracheal aspirate and inoculation site exudates detected only the excretion of *M. bovis* from the inoculation site of one badger. There were marked seasonal variations in body weight but significant weight loss was observed during the second year in all four badgers, particularly prior to death. Four badgers inoculated intratracheally with a similar inoculum of *M. bovis* and another two control badgers showed no evidence of infection with *M. bovis*.

### INTRODUCTION

*Mycobacterium bovis* replicates extensively in badgers and can cause a progressive ultimately fatal disease (Gallagher, Muirhead & Burn, 1976; Little, Naylor & Wilesmith, 1982). Several epidemiological studies have provided convincing evidence that badgers in Britain can be a reservoir of *M. bovis* from which cattle might become infected (Muirhead, Gallagher & Burn, 1974; Barrow & Gallagher, 1981; Little *et al.* 1982; Wilesmith, 1983). It is therefore necessary to identify infected badger populations. Diagnosis of *M. bovis* infection in the badger currently relies upon the isolation of the organism from either faeces, urine or exudates from the respiratory tract, ruptured lymph node abscesses or bite wound lesions of live badgers, or tissues taken at post-mortem examination. Clinical sampling of live badgers detects only 20% of infected badgers (Pritchard *et al.* 1986) and attention has turned to immuno-diagnostic methods. The aim of the present study was to establish a route of experimental infection in badgers which would mimic natural

disease and also provide a model on which to study the immunological response, and measure the efficacy of vaccines.

Clinical and pathological studies on free-living badgers indicate that the route of infection is commonly by bite wounds or via the respiratory tract (Gallagher, Muirhead & Burn, 1976; Pritchard *et al.* 1986).

Francis (1958) and Brown (1983) have reviewed much of the previous work carried out on various routes of infection with tubercle bacilli in experimental animals. Of these methods, the intradermal route has been shown to be useful in comparing the susceptibility of rabbits to tuberculosis as it allows direct measurement of the initial lesion (Lurie, 1941).

The clinical, bacteriological, and pathological findings in badgers experimentally infected with small numbers of *M. bovis* via the intradermal and intratracheal routes are reported in the present study. The immune response of these badgers was monitored by lymphocyte transformation test (LTT) to whole BCG bacilli, skin tests and ELISA tests, the results of which are reported in a succeeding paper (Mahmood *et al.* 1987).

#### MATERIALS AND METHODS

*Badgers.* Badgers were trapped by the staff of the Agricultural Science Service in areas where cattle were free of tuberculosis, and there was no evidence of *M. bovis* in the badgers submitted by the public (Anon, 1984). Badgers were kept isolated in groups known to have shared the same sett, in metal cages with wooden floors and straw beds for 6 weeks. They were then transferred to secure concrete-floored fan-ventilated and heated loose-boxes. Wooden sleeping boxes with straw bedding and a variety of logs and suspended rubber tyres were provided. The animals were fed principally on commercial tinned dog food and biscuits, supplemented with chicken carcasses, eggs and fresh vegetables.

#### *Clinical examination, sampling and bacteriology*

On arrival and at intervals of a month or less, badgers were clinically examined under ketamine anaesthesia (Mackintosh *et al.* 1976). They were weighed, and samples of urine, faeces (using a 'Microlax' enema, Smith, Kline & French Labs. Ltd) and tracheal aspirate together with swabs of bite wounds or other lesions were taken for bacteriological examination. Blood was collected aseptically from the jugular vein for use in LTT and ELISA tests and also collected in EDTA for haematological examination which will be reported separately (Mahmood *et al.* 1987). Badgers were marked using coloured plastic ear-tags and tattooed on the ventral surface of the lower abdomen using Indian ink (Cheeseman & Harris, 1982).

Media used for the examination for mycobacteria included Stonebrinks and Löwenstein-Jensen, with and without pyruvate (Lesslie, 1959), and modified 7H11 agar (Gallagher & Horwill, 1977). Oxalic acid decontamination (Corper & Uyei, 1930) was used for faeces samples and for obviously contaminated tissue samples. Biological tests using guinea-pigs were used only for clinical samples taken following trapping and on post-mortem tissues taken from badgers showing no visible lesions of tuberculosis. The protocol of Huitema (1970) was followed except that 1.25 µg avian PPD and 2 µg of bovine PPD were used to skin test the

guinea-pigs. Organisms were identified as *M. bovis* if they were of typical colonial morphology, strongly acid fast on Ziehl–Neelsen staining and showed enhancement of growth on Löwenstein–Jensen medium with pyruvate. Further confirmatory tests for *M. bovis* were used as described by Marks (1976) and Vestal (1975) with the addition of susceptibility to thiophene-2-carboxylic acid hydrazide (TCH) as described by Vestal (1975). Other mycobacteria were identified as described by Marks (1976).

### *Experimental design*

The dose of tubercle bacilli was based on wet weight and counted using dilution plating (Miles, Misra & Irwin, 1938). A single batch of inoculum was prepared from a recent badger isolate of *M. bovis* (AF 2324/82), by scraping growth from modified 7H11 agar and suspending in sterile phosphate buffered normal saline (PBS, pH 7·2). Two dose levels of 0·01 mg ( $10^4$  bacilli) or 0·001 mg ( $10^3$  bacilli) were used by both intratracheal and intradermal inoculation. The inoculum was checked for viable *M. bovis* by culture and for virulence by guinea-pig inoculation. Badgers were anaesthetized with ketamine hydrochloride and the inoculum was either injected intradermally using a 26-gauge needle in the clipped skin of the medial aspect of the thigh, or flushed with 1 ml sterile PBS into the lower trachea by means of a sterile catheter. Two badgers were used for each dose/route combination. The four badgers injected intradermally (X 3, X 4, X 5, X 6) were housed in one loose box and the four intratracheally inoculated badgers in another (X 7, X 8, X 9, X 10). A control group of two badgers which had received Freund's complete adjuvant 1 year previously were kept under the same conditions in a separate loose box (X 1, X 2).

The experimentally infected and control badgers were observed frequently and monitored by monthly clinical sampling. The survivors were killed with intravenous pentobarbitone sodium under ketamine anaesthesia up to 22 months post-inoculation (p.i.). The intratracheally inoculated group were killed 12 months p.i. and the controls 15 months p.i. Cadavers were autopsied and lesions resembling tuberculosis were taken for cultural and histological examination. Tissues for histology were fixed in 10% formalin, paraffin-wax-embedded and stained with haematoxylin and eosin and Ziehl–Neelsen stains. Four pools of tissues were taken from badgers without visible lesions of tuberculosis, comprising (a) head lymph nodes, (b) lung and associated lymph nodes, (c) kidney, liver, spleen and mesenteric lymph nodes and (d) carcase lymph nodes.

## RESULTS

### *Clinical and bacteriological results*

Guinea-pigs injected with the inoculum showed signs, lesions and skin test responses typical of generalized tuberculosis of the bovine type. Badgers were clinically normal on entry and remained so except for lesions at the site of intradermal inoculation and the draining lymph node which showed some slight enlargement following inoculation. An erythematous papule about 2 mm in diameter was seen in all four badgers 2 weeks p.i. In the group inoculated with

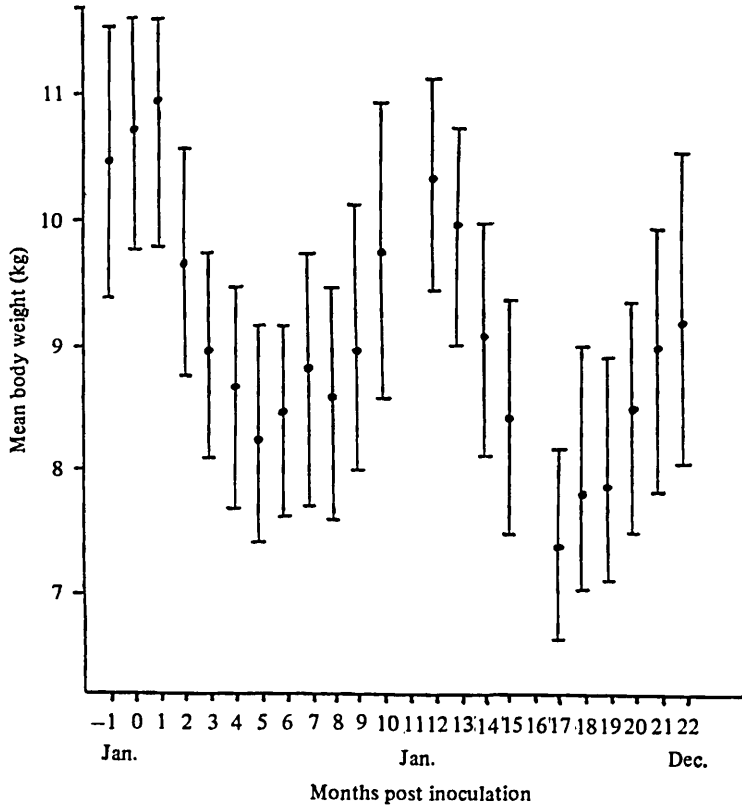


Fig. 1. Mean body weights (kg) of the four badgers inoculated intradermally with *M. bovis*.

$10^4$  bacilli, a necrotic centre appeared at 2 weeks and enlarged to about 5 mm in diameter and slowly healed by 16 weeks p.i. In the group inoculated with  $10^3$  bacilli, the necrotic centre appeared at 3 weeks and progressed to an ulcer, 10 mm in diameter, with an erythematous margin, by 8 weeks. In one of these badgers (X 4) the ulcer healed by 16 weeks, but in the other (X 3), cicatrization was still occurring at 20 weeks. *M. bovis* was isolated from the ulcer of this animal some 16 weeks p.i. but from no other clinical samples.

All badgers showed seasonal variations in weight, being heaviest in December, rapidly losing 15–25% of body weight in April and regaining weight from August to November (Fig. 1). The 2 control and 4 intratracheally inoculated badgers maintained weight in the period post inoculation but 3 out of 4 intradermally inoculated badgers had statistically significantly lower weights in their second year p.i. ( $P < 0.005$ ) than in their first year p.i. Additional weight loss occurred in badger X 3 during the 2 months prior to its death at 17 months p.i.

Two of the sows produced cubs approximately 6 weeks p.i. X 2 in the control group produced five live cubs, all of which were eaten by the mother in the first 24 h. One sow in the intradermally inoculated group (X 5) produced two live cubs, one of which was eaten in the first 24 h, but the second survived for 3 weeks, when it was found dead. It was examined *post mortem* as described previously, with negative results.

Table 1. *Autopsy findings in badgers inoculated intradermally with Mycobacterium bovis*

Badger	Months post-inoculation	Lesions at post-mortem examination	Tissues examined	Isolation of <i>M. bovis</i>
X 3	17 (died)	PM change generalized	Head lns	+
		Lungs – few hard nodules and patchy consolidation	Axillary and inguinal lns	+
		Kidneys – pinhead lesions	Lung and broncho-mediastinal lns	+
		Mesentery – grapes	Kidneys	+
		Blood-filled abdominal cavity	Liver, spleen and mesenteric lns	+
X 4	22 (killed)	Lungs – consolidation and caseation in both apical lobes – few discrete nodules in diaphragmatic lobes	Head lns	–
		Inguinal lns – caseous lesions	Inguinal lns	+
		Spleen and kidneys – pale nodules	Axillary lns	+
		Liver – miliary lesions	Lung and lns	+
		Pleura and mesentery – ‘grapes’	Kidneys	+
		Mesenteric and rectal ln – caseous lesions	Liver	+
			Spleen	+
			Mesenteric lns	+
	Urine	+		
X 5	22 (killed)	Lungs – NVL	Head lns	+
		Bronchial lns – caseous lesions	Inguinal lns	+
		Inguinal lns – caseous lesions	Axillary lns	–
		Kidneys – one pale nodule in left kidney	Lung and lns	+
		Liver – white nodules	Kidneys	–
		Spleen – white nodules	Liver	+
		Mesentery – ‘grapes’	Spleen	+
Mesenteric lns – caseous lesions	Mesenteric lns	+		
X 6	22 (killed)	All organs – NVL	Head lns	–
		Inguinal lns – caseous lesions	Inguinal lns	+
			Axillary lns	–
			Lung and lns	–
			Kidney	–
			Liver	+
	Spleen	–		

NVL, No visible lesions. lns, Lymph nodes

### *Autopsy, bacteriology and histopathology*

No visible lesions (NVL) of tuberculosis were seen at post-mortem examination in the intratracheally inoculated group and the control group. All samples taken from these two groups *post mortem* were negative on biological and cultural tests for *M. bovis*. Badger X 9, which was intratracheally inoculated, showed a few pinhead calcified lesions in the lungs. Histological examination of these lesions revealed foci of silicosis as described by Higgins, Kung & Or (1985) and no acid-fast bacilli were seen. *M. chelonae* was isolated from the broncho-mediastinal lymph nodes, but not from the lung tissue.

The post-mortem findings in the intradermally inoculated badgers are summarized in Table 1. Post-mortem changes had occurred in the organs of badger X 3, which died 17 months p.i., and these rendered it unsuitable for histopatho-

logical examination. Lesions suggestive of tuberculosis were seen in the lungs and broncho-mediastinal lymph nodes of this animal. *M. bovis* was isolated on culture from lungs, liver, spleen, kidneys and broncho-mediastinal lymph nodes and the pools of head and carcase lymph nodes. Badger X 6 showed caseous lesions of the inguinal lymph node draining the inoculation site. *M. bovis* was isolated from this lesion and from the liver. In the other two badgers, typical lesions of tuberculosis, varying from discrete tubercles to several pale or caseous nodules, were present in the lungs, liver, kidney, spleen, and a variety of lymph nodes from which *M. bovis* was isolated. *M. bovis* was also isolated from the urine from badger X 4.

Histopathological examination of the tissues collected at autopsy of the intradermally inoculated badgers showed lesions which varied from many small foci to large areas of lymphocytic and monocytic infiltration with macrophages (but no giant cells) and some necrosis surrounded by epithelioid cells with fibrous encapsulation. The lesions contained few acid-fast bacilli which were present inside the macrophages and epithelioid cells.

#### DISCUSSION

In the absence of a reliable diagnostic test for tuberculosis in live badgers, reliance of freedom from infection was based on capture from areas known to be free of tuberculosis in the cattle and badger populations, and the failure to isolate *M. bovis* from clinical samples on biological tests. In retrospect, the lack of isolation of *M. bovis* by biological and cultural tests from post-mortem material from the control badgers supported this view. The husbandry of the badgers was similar to that used by Little, Naylor & Wilesmith (1982). The badgers adjusted to captivity well and were easy to anaesthetize by intra-muscular injection of ketamine. The provision of logs and hanging tyres provided well-used play facilities and badgers were observed playing, much as described in the wild by Neal (1975). In the absence of soil, the logs were used as scratching posts and claw overgrowth did not occur. The badgers created clearly demarcated activity areas within the loose boxes. A communal sleeping area, either in the wooden box provided or in a straw nest in a corner, was surrounded by a clean feeding and playing area with a dunging area confined to the edges of the two walls of the loose box, centred on the door. Dunging occurred most frequently on top of metal grids covering the drains. Badgers were fed to appetite, and weight gain occurred mainly from August to December, with weight loss in spring. Clear seasonal variations in body weight thus occur in captive badgers independent of food supply, which has been suggested as a major factor in seasonal variations in the weight of free-living badgers (Kruuk & Parish, 1983).

Intradermally challenged badgers showed progressive weight loss in their second year post infection (Fig. 1), which was sudden and considerable in badger X 3. Weight loss is well recognized as a typical presenting sign of tuberculosis in man and domestic animals. Previous studies have demonstrated that, in mammals, metabolic changes occur during bacterial infections, many of which are attributable to products of the reticulo-endothelial system (Dinarelo, 1984). Indeed, mouse macrophages have been shown to secrete the hormone cachectin, which specifically effects enzymes in adipocytes (Beutler *et al.* 1985). This may be the mechanism by which weight loss occurs during the course of tuberculosis.

One of the two sows in the control group cubbed in March, but last had contact with a boar the previous May, thus confirming the unusual delayed implantation features of the reproductive cycle of the European badger (Neal, 1975). The other sow which cubbed was in a group of two sows and two boars. The cannibalism of the cubs 24 h old that was seen in two groups has not previously been described in this species. Cannibalism is well recognized in carnivores and a wide range of other mammals (Fox, 1968).

The aim of inoculating small numbers of tubercle bacilli by intradermal or intratracheal routes was to establish a reproducible model of tuberculosis in the badger, in order to assess candidate diagnostic tests and prophylaxis, using a method which closely mimicked natural infection. Prospective field studies have found that infection via bite wounds is common in badgers (Anon, 1984). It is well established in man and cattle that the major route of spread of tuberculosis is respiratory (Francis, 1958) and the high prevalence of head lymph node and lung lesions in wild badgers (Gallagher, Muirhead & Burn, 1976) suggests that it is of importance in the badger. Intradermal inoculation was used as it has been demonstrated by Lurie (1941) that this route is useful in comparing the relative susceptibility of strains of rabbits to tuberculosis. Exposure of the respiratory tract to aerosols of tubercle bacilli of small particle size has been demonstrated as a highly effective method of infecting animals (Lurie, 1941). As it may result in acute, rapidly fatal, tuberculous pneumonia, this route is unsuited to the study of the immune response over a period of many months, which appears to be the time course of natural infection in both captive badgers (Little, Naylor & Wilesmith, 1982) and wild badgers (Anon, 1984). The use of intratracheal inoculation of tubercle bacilli in a fluid menstruum has been reported in cattle to produce only localized lesions in pulmonary lymph nodes (White & Minett, 1941). This method appeared suited to production of a localized chronic pulmonary tuberculosis in the badger. Wells, Ratcliffe & Crumb (1948) found that large particles containing viable tubercle bacilli failed to establish infection in guinea-pigs. There was no clinical bacteriological or immunological evidence of tuberculosis in the intratracheally inoculated badgers in the present study. The viable bacilli inoculated into the lower trachea may have either been removed by the muco-ciliary escalator or killed by the immune system of the badger. *M. chelonae*, which was isolated from one of the badgers, is commonly found in soil and water and is occasionally the cause of post-injection abscesses in man (Inman *et al.* 1969) and has rarely been isolated from both abscesses and normal lymph nodes of domestic species (Lepper & Corner, 1983).

The development of lesions at the intradermal inoculation site and progressive weight loss was similar to that seen in other mammals (Lurie, 1941; Francis, 1958). It closely followed the course of infection observed in wild badgers (Cheeseman *et al.* 1985) following infected bite wounds, in that suppuration and ulceration occur with enlargement of the draining lymph node. Thus intradermal inoculation with small doses of tubercle bacilli appears to closely reproduce the course of disease seen in infected free-living badgers. There was no evidence of any difference in the response to the dose levels of  $10^3$  and  $10^4$  bacilli used. Difficulties in the estimation of numbers of tubercle bacilli are well recognized, owing to their propensity to clump (Brown, 1983).

A modification of the immune response occurs during pregnancy due to hormonal changes. Badger X 5 was pregnant when inoculated but did not show any clear differences in the course of disease, compared to the other badgers.

Post-mortem examinations and culture revealed that spread of *M. bovis* had occurred by local lymphatics and haematogenously to infect organs such as the kidneys. Infection of the respiratory and alimentary tract may have occurred haematogenously or by the ingestion or inhalation of tubercle bacilli, which were shown to be present during life in suppurating ulcers. Clinical samples were examined by cultural methods only, which recent studies on samples from free-living badgers have shown to have 30% of the sensitivity of biological methods, because only small numbers of bacilli may be present (Pritchard & Stuart, unpublished data).

The results of this study support the evidence of Little, Naylor & Wilesmith (1982) and Cheeseman *et al.* (1985) that infected badgers may survive for several years. Although badger X 3 showed early signs of tuberculous peritonitis and tuberculous pneumonia, these lesions alone did not appear sufficient to be the cause of death. The abdominal haemorrhage was the likely cause of death but its source could not be located, although it may have been related to a tuberculous lesion.

The typical architecture of the tubercle, i.e. giant cells, necrosis and fibrous encapsulation as seen in cattle and man, is not seen in this species. Gallagher, Muirhead & Burn (1976) recorded relatively larger numbers of bacilli in naturally infected badgers, some of which had extensive lesions of tuberculosis. Little, Naylor & Wilesmith (1982) showed that the more extensive lesions in the badgers dying from tuberculosis contained large numbers of acid-fast bacilli. In the present study only small numbers of acid-fast bacilli were seen in the lesions and, except for X 3, the other badgers were killed whilst still in reasonably good condition. Although X 4 and X 5 had tuberculous lesions distributed throughout many organs, the extent of them was not as great as that seen in naturally infected badgers dying from the disease. Thus the relatively few bacilli seen in lesions in these badgers suggests that the multiplication of bacilli in tissues may be successfully limited prior to the development of extensive lesions leading to death.

The results of this study show that the intradermal challenge route is suitable for establishing *M. bovis* infection in badgers, and may prove useful in studying the immune response and in studying the efficacy of vaccination.

The authors gratefully acknowledge the excellent technical assistance of the staff of the TB Section, Bacteriology Department, Central Veterinary Laboratory, and also the work of the staff of the Department of Animal Production in the husbandry of the badgers.

Some of the work described was included in the Ph.D. thesis of K. H. Mahmood (1985).

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