Epidemiol. Infect. (1997), **118**, 173–180

The kinetics of serum antibody responses to natural infections with *Mycobacterium bovis* in one badger social group

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(*Accepted 6 November 1996*)

SUMMARY

Bovine tuberculosis remains a significant problem in some parts of Great Britain and Ireland largely because of a reservoir of infection in badgers. Little is currently known about the immunopathology of *Mycobacterium bovis* infection in the badger. Badgers, from 31 social groups, in a study area of the Cotswold escarpment, have been trapped and sampled from 1981 to 1995. Serum antibody responses directed against the 25 kDa antigen (MPB83) of *M. bovis* have been studied in detail in a selected social group (JM) which has endemic infection. Sequential sera from 44 badgers were studied and results compared with culture from faeces, urine, tracheal aspirates. bite wound swabs and at post mortem. The results indicate that some badgers (about 10%) remain uninfected despite exposure to endemic *M. bovis* infection within the social group. In culture-positive animals active excretion of organisms is not necessarily concomitant with seropositivity. Conversely, seropositivity is not an indicator that culture positivity is present or imminent. This is particularly true in cubs when a transient seropositivity can occur within the first 6–8 months of life but these animals can remain culture-negative for up to 5 years. Western blotting confirms that at least some of these antibodies, detectable by ELISA in the culture-negative cubs, are directed against the 25 kDa *M. bovis* antigen. In contrast antibodies detectable in the culture-positive animals do not Western blot prior to a positive culture. Thus, differential reactivity in Western blotting may distinguish between serum antibodies indicative of potentially culture-positive animals and animals which will remain culture-negative.

INTRODUCTION

Infection of the badger, *Meles meles*, with *Mycobacterium bovis* is now well established in parts of England and Ireland and is believed to constitute the major reservoir of bovine tuberculosis in these areas [1]. Although infected animals can survive for prolonged periods [2] the disease may become rapidly disseminated with excretion of organisms in faeces, urine, sputum and discharging bite wounds. Such excretions probably comprise the routes of transmission to other badgers and to cattle by direct contact or indirectly by contamination of the environment. Recently a serological test was developed to enable diagnosis of disease in live badgers [3]. This test, an indirect ELISA, is being used in an effort to provide strategies for the control of bovine tuberculosis [4]. One strategy currently being trialed is based on the identification of those setts containing serologically positive animals and with subsequent removal of all animals from those setts. Early observations using this test indicate that the sensitivity of detection changes with disease pathology [5] and, possibly, with age of the badger [6].

To date there has been a limited opportunity to study sequential immune responses to pathogenic mycobacteria in naturally infected, untreated, individuals of any species. In humans there would be an ethical requirement for immediate treatment while in cattle there is a statutory requirement for the early eradication of infected animals. Since 1981 sera have been collected from badgers in their natural environment in an area of the Cotswold escarpment in Gloucestershire, England. This area has the highest density recorded for the European badger with 25 adults per square kilometre [7]. Up to 31 badger social groups have been identified in the 9 km² study area. A proportion of this badger population has been shown to have *M. bovis* infection by culture from faeces, urine, bite wounds, tracheal aspirates and post-mortem examination [2]. Serum has been collected from individual badgers at each capture. This data-set therefore provides a unique opportunity to study sequential serological immune responses in a naturally *M. bovis*-infected animal population.

In this report the sera from one social group of badgers were selected for investigation. Antibody levels from sequential serum samples were tested with the indirect ELISA and the antibody specificity determined by Western blotting. The results were correlated with detection of *M. bovis* infection and excretion in these animals over the same period.

MATERIALS AND METHODS

Badger identification and sampling

One social group (Jack's Mirey (JM)) was selected from a well-studied area of the Cotswold escarpment, England, on the basis that (i) a reasonable number of sequential sera had been collected over the period of investigation and that (ii) infection with *M. bovis* had been regularly recorded in animals from this group. Social groups were defined according to a system of territory delineation known as bait marking [8, 9]. Any badger caught within this territory more than once was assumed, for the purposes of this study, to be a member of this social group unless it was trapped in this area on less than 50% of its captures, in which case it was considered transient and excluded from further investigations.

The methods of trapping, handling and sampling have been described elsewhere [2, 10]. Briefly, badgers from this social group were trapped 4 times per year with individual animals being caught an average of 2·1 times per year. Trapped animals were anaesthetized. Faeces, urine and tracheal aspirates, swabs from bite wounds and aspirates from enlarged lymph nodes were collected for bacteriological culture and blood collected for serum.

Bacteriological culture and histopathology

Clinical samples including urine, faeces, tracheal aspirates and swabs from discharging bite wounds were cultured for mycobacteria as previously described [2]. The animal was classified as culture-positive if there was at least one confirmed *M. bovis* culture from any sample at any time from the point of collection onwards. A few of the animals were investigated at post mortem after being found dead for any reason. Any visible lesions were cultured and investigated histologically. The pooled lymph nodes were cultured from animals with no visible lesions (NVL animals) as well as from any animals with visible lesions (VL animals).

Immunological investigations

Blood was collected at each capture. Serum was obtained and stored at −20 °C until required. Serum IgG antibodies were detected using an indirect ELISA as previously described [3]. Briefly, purified 25 kDa *M. bovis* antigen was adsorbed onto micro ELISA plates (0·023 μg/well) and incubated with badger sera (diluted 1 in 10). Badger IgG antibodies attached to the antigen were detected with mouse monoclonal anti-badger IgG [11] coupled to peroxidase [3]. A diagnosis of seropositivity was made on the basis of the 'cut-off' previously described [3] and subsequently modified for field use [5].

Total protein profiles of SDS–PAGE gels of either *M. bovis* sonicate or culture supernatant were analysed by Western blot using badger sera (diluted 1 in 10) as previously described [3].

RESULTS

From the beginning of 1982 to the end of 1994 a total of 47 badgers were trapped in the territory of the selected social group (JM). Three badgers (LO6, N50 and L59) were considered transient animals on the basis of being trapped in this area on less than 50% of trapped occasions. Five additional animals were trapped on only a single occasion. All these animals were considered transients and were therefore excluded from further investigations within this group.

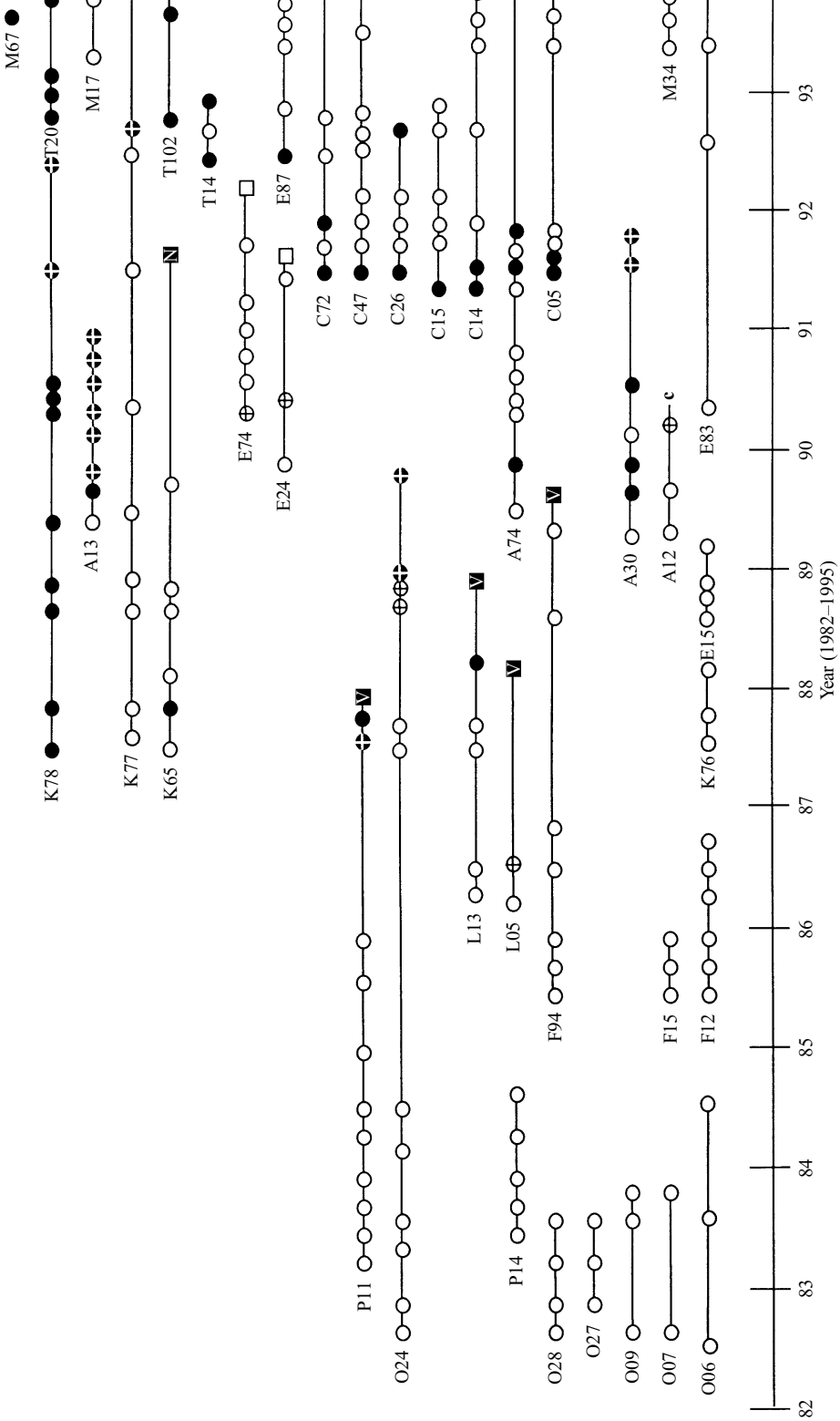

Fig. 1. The results of culture, serological (ELISA) and post-mortem investigations in badgers from the social group JM for 1982–95. Note that the serological data for K78, M17, T20 and Z32 is from original investigations. ●, ELISA positive, culture negative; ○, ELISA negative, culture negative; ⊕, ELISA positive, culture positive; ⊕, ELISA negative, culture positive; □, Post mortem, no visible lesions, culture negative; ▣, Post mortem, no visible lesions, culture positive; ▨, Post mortem, visible lesions, culture positive; C, culture positive, no ELISA done.

Table 1. *The classification of badgers, sampled between 1987 and 1994 within social group JM on the basis of culture and serum ELISA and Western blotting*

Subgroup	Characteristics	Badger numbers
A	Culture negative (or one positive faecal sample with negative post mortem) ELISA negative throughout	M34, E83, K76, E15, E74, E24
B	Culture positive (at least twice) and/or with visible lesions	
	(a) ELISA negative	LO5, A12, F94
	(b) ELISA and Western blot positive at time of culture	O24, L13, P11
	(c) ELISA positive and Western blot negative prior to culture	K77, K65, A30
C	Culture negative	
	(a) Transient ELISA and Western blot positive within 6 months of date of birth	T14, C26, C72, C47, C15, C05, T87, C14
	(b) ELISA positive and Western blot negative	T102, A74

Twenty-eight of the 39 (72%) animals studied from this social group showed evidence of *M. bovis* infection by serology and/or culture (Fig. 1). Until the end of 1985 *M. bovis* infection in this group was absent. In 1986, 1 of 6 animals trapped was culture-positive. However, from 1987 onwards, of the 31 animals sampled only 4 showed no evidence of infection (87% prevalence of infection). During this latter period of endemic infection, 21/31 (67%) animals were seropositive on at least one occasion and 14/31 (45%) culture-positive from at least one clinical sample and/or at post mortem. Two animals (E24 and E74) were serologically negative, negative by culture at post mortem and had only one positive faecal sample each throughout the periods of their sampling (12–24 months). The true infection status of these animals is debatable as these observations are consistent with passage of *M. bovis* through the gut without subsequent infection.

Only 8 of the 28 animals with either serological or culture evidence of infection, in this period, were positive with both methods. Of these cases, seropositivity preceded or occurred at the same time as culture positivity in only four animals. Five animals (E24, E74, LO5, F94 and A12) were apparently actively excreting organisms but were serologically negative throughout their sampling periods (12–48 months). However, E24 and E74 may not have been truly infected (see above).

Eleven of the 21 (52%) serologically positive badgers were culture-negative, from all samples, on at least 3 sampling occasions (number of samples ranged from 2 to 11; sample period 5 months–5 years). Ten (91%) of these culture-negative animals had transient or intermittent seropositivity and in each case the first seropositive sample occurred within 6–8 months of the beginning of their year of birth. Such transient seropositivity only occurred in 2 (K65 and A30) of the 14 (14%) culture-positive animals during this period; in one of these cases (K65) culture was only detected at post mortem (histopathology – no visible lesions detected) 3·5 years after the positive serum sample.

In order to confirm the specificity of the antibody responses, particularly in those animals with transient or intermittent positive serology, 139 sequential sera from 33 selected badgers (21 culture and/or serologically-positive animals and 12 culture and serologically-negative animals (6 of these negative animals were from before 1987)) were tested for reactivity in Western blots against *M. bovis* sonicate and filtrate. Neither the sera from the culture and serologically negative animals, nor ELISA-negative sera from the positive animals, had any reactivity in the Western blots. Of the 35 ELISA-positive sera tested, from 16 animals, 16 were negative by Western blotting. The remainder all detected the 25 kDa (MPB83) antigen in the *M. bovis* sonicate and occasionally in the filtrate. Infrequently the 22 kDa antigen (MPB70) was detected in both antigen preparations.

On the basis of the culture, ELISA and Western blotting results from these selected sera three main subgroups of badgers were distinguishable (Table 1).

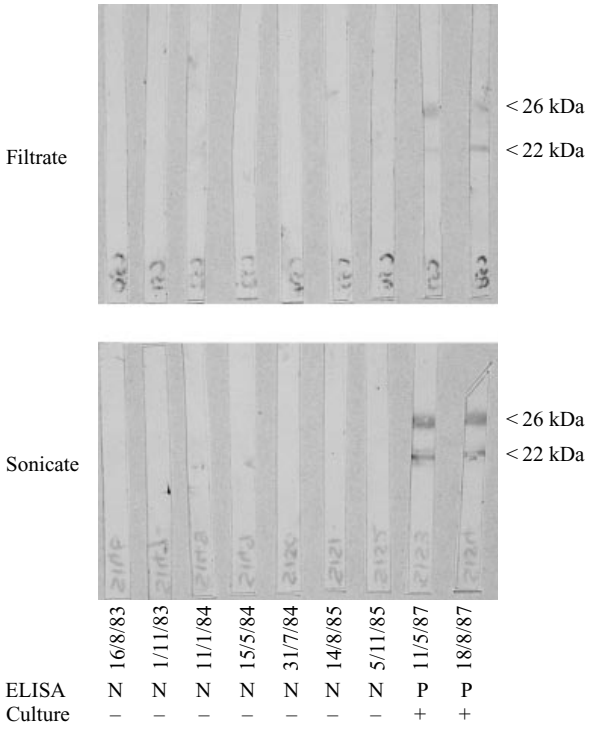

Fig. 2. Western blots of *M. bovis* filtrate and sonicate antigen preparations incubated with sera from badger P11 and correlated with culture and ELISA results. P, ELISA positive; N, ELISA negative.

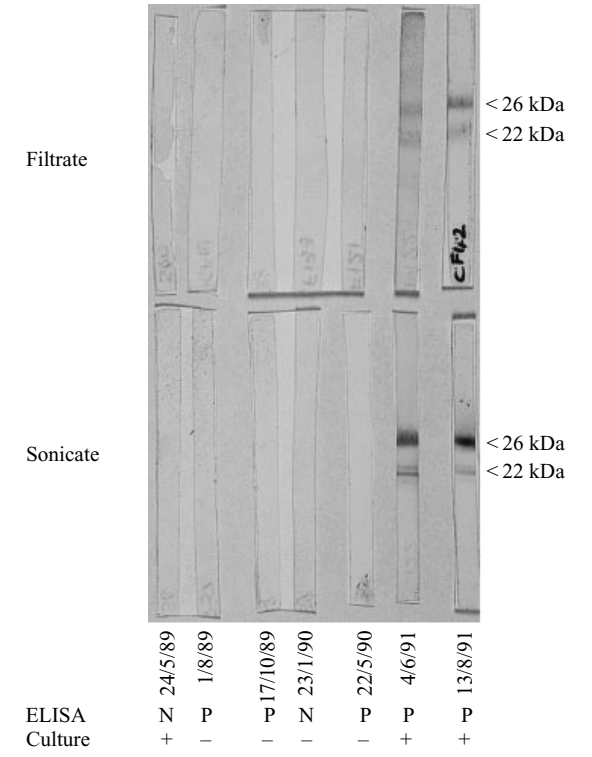

Fig. 3. Western blots of *M. bovis* filtrate and sonicate antigen preparations incubated with sera from badger A30 and correlated with culture and ELISA results. P, ELISA positive; N, ELISA negative.

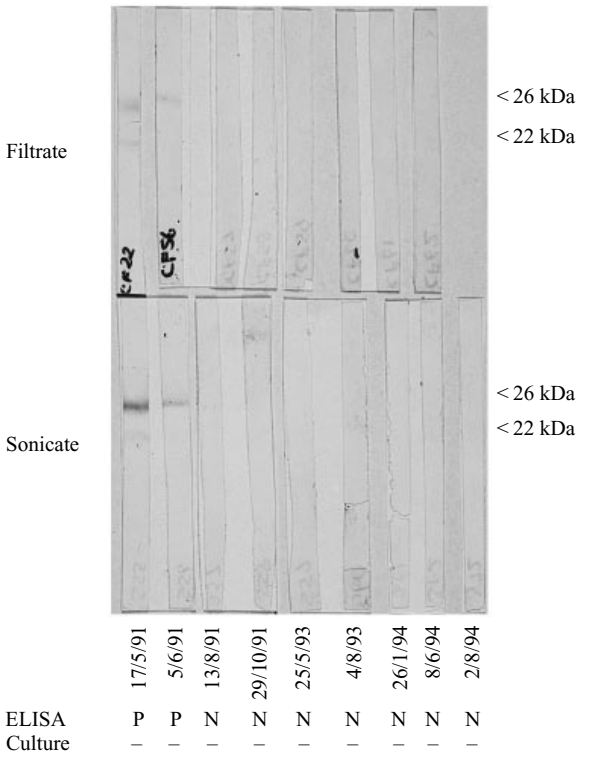

Fig. 4. Western blots of *M. bovis* filtrate and sonicate antigen preparations incubated with sera from badger C05 and correlated with culture and ELISA results. P, ELISA positive; N, ELISA negative.

Subgroup A comprised six badgers which were culture negative, or had one positive faecal sample with a negative post mortem. All sera from these animals were ELISA and Western blot negative. Subgroup B comprised nine animals which were repeatedly culture-positive and/or had visible lesions at post mortem. Three of these animals (subgroup Ba) were ELISA-negative and Western blot-negative throughout. The sera from three animals (subgroup Bb) were ELISA-positive at the time of the first positive culture and those antibodies Western blotted the 25 kDa antigen (Fig. 2). The remaining three animals in this subgroup (subgroup Bc) had ELISA-positive sera prior to the first positive culture, with the exception of A30, but these antibodies did not react in Western blots (Fig. 3). A30 had a single early culture-positive urine sample which was not associated at that time with a positive serological sample but otherwise the animal complied with the pattern described.

Subgroup C comprised 10 animals which were culture-negative but had circulating antibodies detectable by ELISA at some time throughout the trapping period. In eight of these ELISA-positive, culture-negative animals (subgroup Ca) the first (or in

the case of C14 the second) ELISA-positive serum samples all contained transient antibodies which Western blotted primarily against the 25 kDa antigen (Fig. 4) and two of these animals (C26 and T14) had one later serum sample which did not react in Western blots. The two other badgers (subgroup Cb) (A74 and T102), both of which were culture negative, had at least two ELISA-positive, Western blot-negative sera, though the final sample from A74 had a positive Western blot result.

DISCUSSION

Infection with pathogenic mycobacteria is manifest as a spectrum of events distinguishable by a variety of immunological and histopathological states. In particular host immune response to the infection varies over time with cell mediated immunity predominating during the early stages of disease and antibody responses during the latter stages as a consequence of switches in the ratio of Th1 and Th2 cells responding to mycobacterial antigen stimulation [12].

In this study the relationships between *M. bovis* infection and B cell immune responses to this organism have been investigated in one social group of badgers, living in a natural environment, over a 13-year period. The results demonstrate that tuberculosis was endemic within this group after 1986, and, given the social behaviour typical of badgers, it is to be expected that all animals in this group would have been similarly exposed to the infection. Nevertheless, not all the animals became detectably infected as determined by ELISA and/or culture positivity. Moreover, about half of those animals detectably infected did not progress to develop disseminated disease with frank excretion as determined by culture from clinical specimens, such as urine, on at least two occasions or had visible tuberculous lesions at post mortem. This is consistent with previous observations [2] that some badgers survive for long periods with detectable infection and indicates that this species can mount some mechanism, probably immune-mediated, of disease limitation. The nature of this mechanism is currently unknown but past studies suggested that although the cellular immune responsiveness of badgers was poor [13] an antibody responsiveness was detectable [6].

Despite the relatively small number of sera investigated during the endemic period of infection (1987–96) the results suggest that three major subgroups of animals were distinguishable on the basis of infection status and serum antibodies:

Subgroup A included those animals that were negative by all criteria, or were debatably negative accepting a single positive faecal sample with a negative post mortem. About 24% of the animals investigated by all procedures were in this subgroup. However it is, of course possible, that due to the intermittent nature of the trapping a transient positive may have been misclassified.

Subgroup B comprised those animals which actively excreted organisms or had visible lesions suggestive of imminent excretion. In this study these animals constituted about 36% of the whole group. Two-thirds of the individuals from this subgroup were positive in ELISA at some point. One half of these animals were ELISA negative up to the point of culture and then exhibited antibodies detectable by ELISA and Western blotting. The other half demonstrated antibody responses prior to a positive culture but which were only detectable by ELISA.

Subgroup C comprised those animals (about 40%) which did not actively excrete organisms but had an ELISA-positive sera, within 6–8 months of the presumed date of birth. Eight of these sera also contained antibodies detectable by Western blotting. These animals quickly became seronegative, sometimes within a few weeks, though some had intermittent seropositivity thereafter. It seems likely that at least some of the animals from subgroup A may also have fallen into this latter subgroup since the sampling regime may have missed a transient seroconversion.

The results obtained in this study indicate that the presence of antibodies, detectable by the ELISA, in young animals does not necessarily correspond with *M. bovis*-culture positivity. One possible explanation is that the ELISA is not specific. However, this seems unlikely as laboratory and field trials [3, 5] indicate that the specificity of this assay is greater than 95%. However, recent analysis of field trial data suggests that the specificity at the population level [5] decreases from 83·6% (72·7–90·7) when adults only are included to 79·5% (68·1–87·7) when cubs are also included, suggesting that there may be a disproportionate number of cubs which are culture-negative but ELISA-positive (Clifton-Hadley, unpublished data). Even though the specificity of the ELISA against the 'gold standard' of culture may be lower in cubs than adults, the antibody reactivity detected is directed against the 25 kDa *M. bovis* antigen as shown by

Western blotting, indicating exposure to the organism within the sett. Therefore these results still support the continued current use of the indirect ELISA in control strategies [1] as the objective is to remove infected setts regardless of the badger's immune status.

Given that all the animals in subgroup C were first trapped as young cubs and gave serological responses at the first or second capture while probably less than 6–8 months old, one explanation of these results could be the presence of maternal antibodies placentally or passively transferred from an infected dam. Pseudo-vertical transmission has been considered a potentially significant route of infection within setts [14] and such antibodies would also be a strong indicator of sett infection in the development of control strategies. Unfortunately current technology does not allow distinction between maternal and cub antibodies. The recent development of fingerprinting techniques for badgers (T. Burke, University of Leicester, personal communication) may enable family relationships within social groups to be analysed and establish the source of such antibodies in the future.

Over the period studied none of the animals from subgroup C showed evidence of infection whereas animals from subgroup B became clearly culture-positive and usually infectious. Follow-up of subgroup C animals through 1995 indicates that only one animal (C47) has subsequently become detectably infected. If the transient seropositivity observed in subgroup C animals is a true reflection of infection then these animals appear to have an extended period of latency. Until such animals are available for post-mortem examination their true infection status will remain unknown. The source or role of these antibodies is currently unknown. However, it is evident that ELISA-positivity in cubs is not necessarily an indicator of disease associated with concurrent or imminent excretion of organisms. The possibility that such transient seropositivity in young badgers may be indicative of protection from disease cannot be excluded and deserves further investigation in other social groups of badgers with endemic disease. If this observation is confirmed then the effect of the removal of such cubs on the genetic basis of disease resistance within social groups would also need to be investigated.

One distinction between animals from subgroups B and C is the immunochemical properties of their detectable antibodies. Serum antibodies observed, prior to culture-positivity, in animals from subgroup B were primarily detectable only by ELISA whilst those from subgroup C were primarily detectable by both ELISA and Western blotting. A possible explanation is that in the process of SDS–PAGE prior to Western blotting the antigen is denatured resulting in the loss of three-dimensional structures and, therefore, of conformational epitopes. These results suggest that those IgG antibody responses, detectable by ELISA only, appear to be directed solely against the conformational epitopes of the 25 kDa antigen, whereas other animals exhibited antibodies with specificity for linear epitopes, present in both the 25 and 22 kDa antigens and detectable by Western blotting.

Recently the antigenic relationship between the 25 and 22 kDa antigens has become clearer. Previously it was thought that the 25 kDa antigen was a glycosylated form of the 22 kDa antigen, MPB70 [15]. However, the 25 kDa antigen is now assumed to be the glycosylated product of the distinct gene, MPB83. In *M. tuberculosis* the homologue of this gene MPT83 has 65% DNA homology with the MPT70 gene [16]. Such an association would account for the antibody cross-reactivity between the two proteins on Western blotting. At this time there appears to be no obvious relationship between the specificity of the antibodies detected and disease status in this group of animals. However, the number of Western blot-positive sera investigated to date is too small for more detailed analysis.

One possible interpretation of these data is that the presence of antibodies that are both transient antibodies and directed against the linear epitopes of the MPB83 antigen is indicative of protection of badgers from active disease later in life. Previous studies [12, 17], using experimental vaccine and challenge models in mice and guinea-pigs, clearly show that immune protection against tuberculosis is mediated by T cell responses and that the presence of antibody responses is thought to indicate disease progression presumably as antigen from extracelluar organisms is disseminated. Nevertheless, the presence of some antibodies may indicate exposure to mycobacterial antigens with subsequent induction of a transient B cell response and a long-lived and effective T cell response. The investigation of antigen-specific T cell responses in badgers within social groups with endemic disease would enable further understanding of the immune status of these animals.

This study indicates that at least some badgers develop transient antibody responses associated with extended periods of latency or possibly even pro-

tection from progressive disease. It must be emphasized that these observations are based on a relatively small sample and are not necessarily representative of the whole study area or of other, geographically distinct populations of badgers. Further investigations in four other social groups within the study area are currently in progress. Nevertheless, should these observations be confirmed then it provides optimistic evidence that the development of a vaccine against badger tuberculosis is a potentially feasible strategy [18].

ACKNOWLEDGEMENTS

We would like to thank Ms W. Pressling and Ms N. Commander for performing the laboratory assays, the field team (P. Mallinson, J. Howell, P. Spyvee and D. Handell) responsible for the collection of samples and the TB Culture Unit (CVL) (especially N. Palmer, P. Croston and C. Lock). This work was funded by the Ministry of Agriculture, Fisheries and Foods, GB.

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