

## The immunological consequences of challenge with bovine tubercle bacilli in badgers (*Meles meles*)

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### SUMMARY

Optimal conditions were determined for performing antibody measurements (ELISA), lymphocyte transformation tests and, to some extent, skin tests in badgers. These parameters, together with the bacteriological and pathological studies reported previously (Pritchard *et al.* 1987), were used to follow the course of intradermal and intratracheal challenge of badgers with bovine tubercle bacilli. Two challenge doses were used for each route of infection and two animals received each dose. None of the four animals challenged by the intratracheal method showed any evidence of infection, suggesting that adult badgers may have some resistance to challenge by this method. All four animals challenged intradermally developed lesion of tuberculosis.

Immunologically the disease passed through three phases. There was an early phase in which lymphocyte transformation to whole BCG steadily and significantly increased, and skin tests to tuberculin became positive but there was little change in antibody levels. This was followed by an intermediate phase of variable skin responses, fluctuating lymphocyte transformation and significant increase in antibody levels. The final phase, which was only seen in two animals with extensive disease, was associated with changing skin reactions and falling lymphocyte responses, together with a sudden increase in antibody levels.

This paper presents the first formal evidence of cell-mediated immunity to tuberculosis in the badger, which may delay onset and prolong the survival of challenged animals.

### INTRODUCTION

As described previously (Muirhead, Gallagher & Burn, 1974) badgers infected with bovine tubercle bacilli may act as a reservoir of infection for cattle. There are difficulties in detecting infection in the live badger and attention has turned to immunological methods of diagnosis. Serological tests such as enzyme-linked immunosorbent assay (ELISA) have demonstrated that badgers produce antibodies to mycobacteria but are not useful as diagnostic tests due to low specificity

(Morris *et al.* 1979). Interestingly, Higgins & Gatrill (1984) found that antibody responses in badgers to a variety of antigens were low in comparison with those of rabbits.

Microscopic and histological examination of lesions has provided little evidence of cell mediated immunity (CMI) comparable to that seen in some other mammals (Gallagher *et al.* 1976). Little *et al.* (1982) and Morris *et al.* (1978) found that delayed (Type IV) hypersensitivity skin responses and *in vitro* lymphocyte transformation responses (LTT) to purified protein derivative (PPD) of bovine tubercle bacilli were invariably negative whether badgers were uninfected, naturally infected or experimentally infected. Corbel *et al.* (1983) found that delayed-type hypersensitivity (DTH) responses to *Brucella abortus* antigen in badgers infected with this organism were poor. Higgins (1985) found that PPD stimulated a small increase in skin thickness with a timing consistent with DTH response in naturally infected badgers. Neither erythema, palpable oedema nor induration were seen but a distinct histological reaction was present.

This paper reports the immunological findings in badgers experimentally infected with small numbers of bovine tubercle bacilli via the intradermal and intratracheal routes. The immune responses of these badgers was monitored by lymphocyte transformation tests to whole BCG bacilli, skin tests and ELISA tests.

#### MATERIALS AND METHODS

The details of the experimental design and methods are described by Pritchard and others (1987). Ten badgers were housed in two groups of four and one group of two control animals (X 1 and X 2) which had received Freund's complete adjuvant in an earlier study.

All the animals had been in captivity for at least 12 months prior to the start of the experiment, during which time tuberculosis had not been detected by clinical sampling in any of the badgers. Optimum conditions for the LTT and for ELISA for anti-mycobacterial antibodies were determined and baseline values were obtained for each animal.

The two groups of four badgers were challenged with either 0.01 mg ( $10^4$  bacilli) or 0.001 mg ( $10^3$  bacilli) wet weight of bovine tubercle bacilli, either intradermally (badgers X 3, X 4, X 5, X 6) or intra-tracheally (badgers X 7, X 8, X 9, X 10). Two animals thus received each dose/route combination.

The badgers were sampled at monthly intervals under ketamine anaesthesia (Mackintosh *et al.* 1976). Blood samples were collected aseptically from the jugular vein into two glass universal containers, and defibrinated by shaking with glass beads for use in LTT and ELISA. All surviving badgers were killed under ketamine anaesthesia with intravenous sodium pentobarbitone, the intra-tracheal group at 12 months post-inoculation (p.i.), the controls after 15 months, and the intradermal group at 22 months p.i.

#### *Lymphocyte transformation test (LTT)*

Mononuclear cells were prepared from the blood samples as described by Böyum (1968). Whole live BCG (Glaxo) organisms were used as the stimulating antigen prepared as described by Rook *et al.* (1985). Optimal conditions for carrying out

LTT on badger blood samples were determined in a series of preliminary experiments prior to experimental challenge of the animals. It was found that the best results were obtained if the microtitre plates were pretreated at 37 °C for 30–60 min with medium containing 10% of autologous serum. Then  $0.8 \times 10^6$  cells per well were cultured in the same serum concentration.  $1 \times 10^6$  whole live BCG organisms were used as the stimulating antigen and cultures were maintained for 5 days. The ranges of each of the parameters from which optimal concentrations were selected were (1) coating of microtitre plates with 5%, 10% and 20% serum, (2) cell densities of  $1 \times 10^4$  to  $0.8 \times 10^6$  per well, (3) BCG  $5 \times 10^4$  to  $4 \times 10^6$  per well, (4) incubation periods of 2–5 days. Results were analysed using analysis of variance (Snedecor & Cochran, 1971).

### ELISA test

Rabbit anti-badger immunoglobulin (Ig) was prepared according to the method of Voller *et al.* (1979) and conjugated with horseradish peroxidase using the modified method of Wilson & Makane (1978). The solid phase ELISA was conducted using the principles outlined by Nassau *et al.* (1976).

Optimal conditions for the test sera were found from checkerboard titrations to be: 10 µg/ml of antigen for coating the wells at 4 °C overnight; 1/200 dilution of the test sera; 1/800 dilution of horseradish peroxidase labelled rabbit anti-badger Ig. The substrate used was 2,2-azino-D(3-ethylbenzthiazoline sulphonic acid) and hydrogen peroxide. The antigens used were New Tuberculin (NT), Aviumin-B (AB) and Vaccin (V) prepared from *Mycobacterium tuberculosis*, *M. avium* immunodiffusion type B and *M. vaccae* respectively, according to Paul, Stanford & Carswell (1975). Purified protein derivative (PPD) from bovine tubercle bacilli was supplied by the Biological Products and Standards Department, Central Veterinary Laboratory, Weybridge.

The results of antibody absorbance were expressed as mean optical density (OD) of duplicates. A positive control serum was included in each set of tests and OD's of each were corrected according to the mean OD of the positive control in the first run divided by the mean OD of positive control in each test run. Results were analysed using analysis of variance (Snedecor & Cochran, 1971).

### Skin tests

Prior to experimental infection the badgers were skin tested with New Tuberculin (NT), Aviumin B (AB), bovine PPD, a pool of sonicates of 12 slow-growing (SG) mycobacteria and a pool of sonicates of 12 fast-growing (FG) mycobacteria as described by Stanford *et al.* (1981), each at 0.2, 2 and 20 µg/ml in 0.1 ml sterile borate-buffered saline (pH 8.0). The reagents were injected intradermally into the clipped chest wall over the posterior rib cage. The test sites were examined after 72 h and any induration, oedema and/or erythema were measured and recorded.

All the initial test results were negative, as were further tests on the same badgers either incorporating equal amounts of cortisone acetate (25 mg/ml) with the same reagents or using a second injection of reagents into the same site 72 h after the first injection.

After challenge, skin testing was carried out at 3-monthly intervals using 2, 20 and/or 100 µg/ml of NT and PPD and read at 72 h. Biopsies of positive reactions to PPD were taken on two occasions under anaesthesia for histopathology.

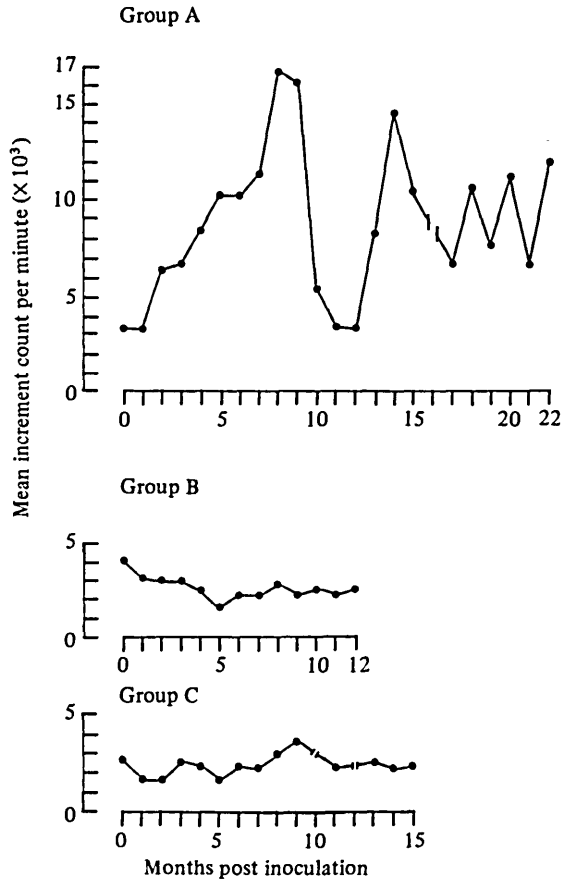


Fig. 1. LTT results of badgers challenged with bovine tubercle bacilli intradermally (group A), intratracheally (group B), and the control group (C).

## RESULTS

### *Lymphocyte transformation test*

No significant increased transformation in response to BCG (Glaxo) was seen in the control or intratracheally inoculated badgers. The intradermally inoculated badgers showed a significantly increased response at 2 months ( $P < 0.001$ ) which continued to increase until 9 months p.i. (Fig. 1). A decline in the response was seen in months 10–12 and then it increased to a high level 14 and 15 months p.i. In the three surviving badgers the transformation response was highly variable between months 15 and 22 p.i.

### *ELISA results*

Analysis of variance revealed small but statistically significant increases in optical densities (OD) to all four antigens used in the ELISA test during months 1–11 p.i. in the intradermally challenged group. There were significant differences between antigens. There was an increasing gradation of OD from PPD to Vaccin to Aviumin-B to New Tuberculin. The intradermally challenged group continued to show marked increases in OD to all four antigens from month 12 to month

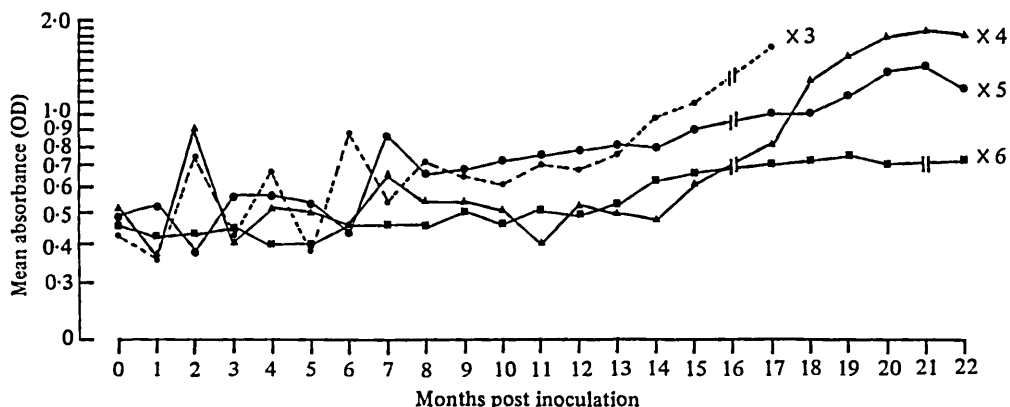


Fig. 2. ELISA results of the badgers challenged intradermally with bovine tubercle bacilli using new tuberculin as the antigen.

22 p.i. There was marked individual variation but those three badgers with generalized tuberculosis had greater increases in antibody than the badger with localized lesions. Fig. 2 illustrates these changes in ODs to New Tuberculin.

No increase in OD to any of the four antigens were detected in the intratracheal or the control groups. Skin testing did not have any significant effect on the anti-mycobacterial antibody levels measured.

#### *Skin test results*

Oedema and erythema with palpable induration up to 15 mm in diameter were seen, for the first time, in intradermally inoculated badgers when tested 3 months p.i. These reactions were not seen consistently and varied with antigen type and concentration (Table 1). Badger X 3 was not tested until 9 months after challenge, when it was found to react to both NT and PPD. This persisted 12 months after challenge, but by 15 months reactivity was lost to NT although still present to PPD. The animal died prior to the next test date. Badger X 4 responded to both reagents 3, 6, 9 and 15 months after challenge, was negative to both reagents at months 12 and 18, but PPD responsiveness returned alone at month 21. Badger X 5 responded to both reagents at 3, 6, 9, 18 and 21 months post-infection, but reacted to PPD only at months 12 and 15. Badger X 6 was not tested at months 3 and 6 and was negative to both reagents at 9 and 12 months after challenge. This animal never reacted to NT, but produced positive responses to PPD from month 15 onwards.

Histological examination of biopsies of the oedematous or erythematous reactions to PPD taken at 72 h after the skin test showed a predominantly lymphocytic infiltration and many of the aggregated macrophages were elongated resembling epithelioid cells but no giant cells were seen.

In contrast, all the animals in the control and intratracheally challenged groups were invariably unreactive to the reagents at all concentrations used for skin testing prior to, and after, challenge.

#### *Clinical sampling and autopsy results*

These are described in detail in the previous paper (Pritchard *et al.* 1987).

Table 1. Results of badgers inoculated intradermally with bovine tubercle bacilli and skin tested at 3-monthly intervals post inoculation with New Tuberculin (NT) and Bovine PPD

| Months after challenge | Reagents ( $\mu\text{g/ml}$ ) | 72 h skin reactions (mm diameter) |       |         |      |
|------------------------|-------------------------------|-----------------------------------|-------|---------|------|
|                        |                               | X 3                               | X 4   | X 5     | X 6  |
| 3                      | NT2                           | ND                                | 8     | 10      | ND   |
|                        | PPD2                          | ND                                | 11.5  | 13      | ND   |
| 6                      | NT2                           | ND                                | 6     | 7       | ND   |
|                        | NT20                          | ND                                | 13    | 12.5    | ND   |
|                        | PPD2                          | ND                                | 7     | 10      | ND   |
|                        | PPD20                         | ND                                | 15(b) | 14.5(b) | ND   |
| 9                      | NT2                           | 0                                 | 8     | 8       | 0    |
|                        | NT20                          | 7                                 | 8     | 8       | 0    |
|                        | PPD2                          | 3.5                               | 6     | 6       | 0    |
|                        | PPD20                         | 10                                | 10    | 10      | 0    |
| 12                     | NT20                          | 5.5                               | 0     | 0       | 0    |
|                        | PPD20                         | 13                                | 0     | 11.5    | 0    |
| 15                     | NT20                          | 0                                 | 13.5  | 0       | 0    |
|                        | PPD20                         | 13                                | 15    | 15      | 3.5  |
| 18                     | NT20                          | ND                                | 0     | 6       | 0    |
|                        | NT100                         | (died)                            | 0     | 10      | 0    |
|                        | PPD20                         |                                   | 0     | 9       | 12.5 |
|                        | PPD100                        |                                   | 0     | 12      | 14   |
| 21                     | NT20                          | ND                                | 0     | 0       | 0    |
|                        | NT100                         |                                   | 0     | 6       | 0    |
|                        | PPD20                         |                                   | 9     | 9       | 11   |
|                        | PPD100                        |                                   | 11(b) | 13(b)   | 12   |

ND. Not done; (b), biopsies were taken for histopathology; 0, no reaction.

In summary, bovine tubercle bacilli were isolated from the ulcerated intradermal inoculation site of badger X 3, 16 weeks p.i. All other clinical samples were negative for tubercle bacilli. Badger X 3 died at 17 months post-inoculation.

At post-mortem examination, lesions of generalized tuberculosis were seen in badgers X 3, X 4 and X 5, and bovine tubercle bacilli were isolated from many tissues and lymph nodes. Badger X 6 showed only caseous lesions of the inguinal lymph node draining the inoculation site, from which bovine tubercle bacilli were isolated. The liver was also positive on culture. The intratracheally challenged and control badgers showed no visible lesions of tuberculosis and were negative on biological and cultural tests for tubercle bacilli. *Mycobacterium chelonae* was isolated from the bronchial lymph node of badger X 9.

#### DISCUSSION

This study required badgers which were free from tuberculosis and they were trapped in areas which had been free from known bovine cases of tuberculosis for many years. The lack of good diagnostic tests for tuberculosis in live badgers means

that some of the badgers could have been infected prior to trapping. However, the failure to detect bovine tubercle bacilli by both cultural and biological tests from the clinical samples taken from all animals prior to the start of the experiment, together with the significant increase in the level of *in vitro* lymphocyte stimulation by BCG and positive skin test reaction in the intradermally challenged group after challenge compared with the lack of response in the other badgers, gives some assurance that they were initially free from infection.

In order to monitor cell-mediated and humoral immune responses during the course of the experimental infection, and as data on suitable test systems were not available at that time, optimal conditions for LTT, ELISA and, to some extent, skin tests, were elucidated during the study. Whole live BCG were found to be suitable for LTT with badger cells. Soluble extracts of mycobacteria were usually toxic. This may explain the previously published failure to demonstrate LTT responses in badgers using PPD as the antigen (Morris *et al.* 1978).

The intratracheal challenge route failed to lead to tuberculosis in all four animals and these animals behaved as did the controls. The infection of badger X 9 with *M. chelonae* did not result in any response in the immunological tests used. The four intradermally challenged badgers all developed tuberculosis accompanied by increases in lymphocyte transformation, increase in anti-mycobacterial antibodies and by positive skin tests. This group cannot be readily compared with the usual laboratory animals, since they were caught from the wild, were of different ages, and undoubtedly had different experiences of mycobacteria prior to their capture. Thus they were much more individual in their responses to challenge. Despite all these complications, a general pattern of response to intradermal challenge with virulent bovine tubercle bacilli has been established, around which each animal had its own variations.

As a group, the intradermally challenged badgers showed a statistically significant ( $P < 0.05$ ) increase in their *in vitro* LTT starting 2 months after challenge and reaching a maximum in the eighth or ninth months (early stage). Clinically positive skin-test reactions to both NT and PPD have been observed for the first time in three of the four badgers at the times they were tested during the first year after challenge. In a previous report of experimental infection of badgers using the intravenous route, no positive skin-test reactions were seen (Little, Naylor & Wilesmith, 1982). The badgers showed no statistically significant increase in their anti-mycobacterial antibody levels during this stage of the experiment. Thereafter, during the period between 9 and 20 months after challenge (intermediate stage), the *in vitro* LTT levels started to decline and then showed a seesawing decrease and increase. The skin-test reactions to PPD remained, and in the case of X 6 became positive for the first time, but were less consistent than in the early stage. Reactivity to NT never developed in X 6, disappeared during the intermediate stage in X 3 and X 4, and became variable in X 5. The animals started to show a marked increase in their antibody levels during their intermediate stage that became statistically significantly raised at month-17 and onward. This late stage was only seen in two of the animals during the course of the experiment. This was in the 2 months prior to death (X 3) or shortly before termination of the experiment (X 4), when these animals showed very low LTT, poor or negative skin tests and significantly higher anti-mycobacterial antibody levels than in the

intermediate stage. Both of these animals showed typical lesions of generalized tuberculosis and bovine tubercle bacilli were isolated from the organs and lymph nodes on culture. Another badger, X 5, showed generalized disease at the end of the experiment, but this had progressed somewhat less and the late stage of the immunological changes was not reached. The final animal (X 6) showed low LTT, good skin test reaction and very low antibody levels as compared to its initial values at month 0. This badger showed no visible lesions of tuberculosis on post-mortem examination except for caseous inguinal lymph nodes; bovine tubercle bacilli were isolated from these nodes and from the liver only. The differences in responses to NT and PPD after the initial phase are interesting and suggest that the antigens to which the reaction is directed or the regulation of responses to the two reagents may be different.

The observation that antibodies to *M. vaccae* (V), *M. avium* (AB) and *M. tuberculosis* (NT) all increase similarly suggests that most antibodies are directed towards group i antigens (Stanford, 1983). These are the common mycobacterial antigens, and the only ones shared by all three species. Although it is difficult to directly compare antibodies to PPD with those to sonicate preparations, the increase in antibodies to PPD (bovine) suggests that some of them may be to group ii (slow-grower-associated) or group iv (species-specific) antigens. This increase in antibodies to common antigens is usually seen in man with tuberculosis and has been a major problem in the search for a diagnostic serological test for the disease.

The present study illustrates some important features of the immune response in badgers challenged with bovine tubercle bacilli. In the initial phase of disease the cell-mediated immune response against tubercle bacilli was well developed and the antibody response to mycobacterial antigens (NT, AB, V, PPD) was slightly raised. In the intermediate stage there were signs of failure in immune control of the infection, with faltering lymphocyte responses, weaker skin-test responses and increasing antibody levels. In the late stage seen in badger X 3, depressed cell-mediated responses, rocketing antibody levels and falling body weight heralded death with generalized tuberculosis. Thus it appears that badgers show a range of immune responses to bovine tubercle bacilli, during the course of the disease as described in other species (Thorns & Morris, 1983).

Four tentative, but important, conclusions can be made from this study: first, although there were only four animals in each challenge group it is striking that intradermal challenge was always successful and intratracheal challenge always failed, suggesting that adult badgers may be relatively resistant to an intratracheal route of infection but susceptible to bite-wound infection. The aerosol route has not yet been investigated. Secondly, badgers appear to mount immune responses to tuberculosis, apparently holding the disease in check for a variable period of time; in fact, for the entire 22 months of the study in the case of animal X 6. Thirdly, the animals are only likely to be infectious to other badgers, or a potential danger to cattle, in the late stage when their immune mechanisms have failed and they shed bacilli as demonstrated by badger X 4. Fourthly, and most speculative, if a vaccine could be developed that would enhance cell-mediated immunity, substantially prolonging the early and intermediate phases of the disease, the chain of infection might be significantly broken.



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