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Mycobacterium vaccae and immune responses: implications for leprosy control

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Summary *Mycobacterium vaccae*, common in some tropical environments, may have a beneficial effect on the incidence of leprosy by acting as a natural vaccine, or have a real or apparent harmful effect by interfering with the protection afforded by BCG vaccination. We are using an animal model to assess these possibilities. The results reported here show that a strain of *M. vaccae* isolated from Ugandan mud can evoke a significant immune response in mice sensitized subcutaneously or orally. Spleen cells from such mice responded equally well *in vitro* to *M. vaccae* and BCG as measured by two independent assays. Good responses were observed for at least 3 months after oral exposure to *M. vaccae* even though no viable organisms could be detected in the organs at this time showing that persistence of *M. vaccae* is not necessary for expression of sensitization. These experiments support the idea that people who become sensitized to *M. vaccae* or certain other environmental mycobacteria might be expected to show some resistance to leprosy. However, BCG vaccination might appear ineffective in that many individuals would already be sensitized to antigens common to all mycobacteria.

Introduction

The presence of *Mycobacterium vaccae*, a non-pathogenic organism common in certain environments especially those in tropical countries, may have important implications for the incidence of leprosy and its control. The suggestion has been made that *M. vaccae* may act as a natural immunogen.[1] The evidence in support of this view relies largely on skin test positivity in certain communities towards an extract prepared by sonication of *M. vaccae* (vaccin) and the incidence of leprosy in these communities.[1] Further, the pattern of these delayed skin responses to vaccin is similar to that to extracts of *M. leprae* (leprosin A) in that the responses of contacts of leprosy patients to leprosin A often correlate closely with their

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responsiveness to vaccin[2],[3] and in leprosy patients themselves anergy to leprosin A often correlates with anergy to vaccin.[4]

It has been suggested[1] that superimposing BCG vaccination in Uganda upon a presumed natural vaccination resulting from the exposure of the population to *M. vaccae* or other fast-growing mycobacteria in the environment provided an additive protective effect and was responsible for the 80% efficacy of the BCG vaccination trial there against leprosy.[5] In areas of Burma, however, where a BCG vaccination trial was only 20% effective,[6] the synergistic effect of such organisms was postulated to have been negated as a result of exposure to another environmental mycobacterium, *M. scrofulaceum*.[1] The possibility also exists that the presence of mycobacteria such as *M. vaccae* in leprosy endemic areas where BCG vaccination has appeared to fail may have affected the results because BCG vaccination provides no additional benefit above that provided by the 'natural' immunogen. Such an effect was shown to occur when mice or guinea-pigs were exposed to certain atypical mycobacteria (such as *M. avium*), as well as being vaccinated with BCG, in experimental tuberculosis.[7,8] An alternative possibility (as yet uninvestigated) is that *M. vaccae* might interfere with the host's natural immunity to leprosy and/or responses to the BCG vaccine.

To date, animal studies have generally argued against a significant role for *M. vaccae* as they have suggested that the species is poorly immunogenic[9,10] with a very limited ability to protect mice from a challenge with viable *M. leprae*.[11] However, these studies used a limited representation of the strains of *M. vaccae* and strain differences within a species of mycobacteria have been shown to produce very different immune responses in the murine host.[12] We report here some studies on the sensitization of mice with a strain of *M. vaccae* isolated directly from the mud of the BCG vaccination trial area in Uganda.

Materials and methods

MICE

Eight- to 12-week-old female CBA/Ca mice bred at St Mary's Hospital Medical School were used. Animals to be injected subcutaneously (s.c.) with the bacilli were conventionally fed and housed and given tap water to drink *ad libitum*. For these experiments no measures were taken to reduce their exposure to other mycobacteria. Animals to be fed with the bacilli were suckled conventionally but placed into 80-mice sterile isolator units (Olac 76, Bicester, Oxon) and offered sterile food and distilled water *ad libitum*, immediately on weaning. The animals were maintained in this environment throughout the experimental period to inhibit their exposure to other mycobacteria.

MYCOBACTERIA

Mycobacterium vaccae (strain R877R)

We used a stable rough variant of a strain isolated from Ugandan mud by Dr J Stanford, Middlesex Hospital Medical School, London. Bacilli were grown on Sauton agar at 37°C for 2–4 weeks. Suspensions were prepared in sterile water and the total concentration of organisms was estimated by nephelometry. The viability of the suspensions determined retrospectively by colony counts on Sauton agar was $< 5\%$.

Mycobacterium bovis, BCG

For injection, 50-dose ampoules of lyophilized BCG (batch C390; Glaxo Laboratories Ltd, Greenford, Middlesex) containing $1{\cdot}4 \times 10^8$ total (7×10^7 viable) organisms were reconstituted with 0·45 ml sterile water.

For use in *in vitro* assays, BCG was grown in bulk in glycerol-free medium (Glaxo Laboratories Ltd). The organisms were harvested and total numbers determined as for *M. vaccae*. Suspensions of both organisms were heat-killed by incubation at 70°C for 1 h.[13] The dead organisms were washed in tissue culture medium and resuspended to the desired concentration before use.

ADMINISTRATION OF MYCOBACTERIA TO THE MICE

Subcutaneous route

Mice were injected s.c. in the right rump with 0·2 ml mycobacterial suspension. Each mouse received a total of 10^8 *M. vaccae* (2×10^6 viable) or 6×10^7 BCG (3×10^7 viable).

Oral route

To investigate the effect of feeding the mice with *M. vaccae*, 8-week-old mice in isolator units were given sterile distilled water deliberately contaminated with *M. vaccae*. The mice were fed for 3 continuous weeks, the water replaced with a freshly contaminated preparation each week. The average concentration of *M. vaccae* in the water over the 3 weeks was 10^8 total (2×10^6 viable) organisms per ml water. Age- and sex-matched mice maintained in a separate isolator unit under identical conditions but without administration of the mycobacteria were used as controls.

DETECTION OF VIABLE MYCOBACTERIA IN MOUSE TISSUES

Samples of spleen, liver and lung were homogenized in 5 ml of distilled water (2

min, Colworth Stomacher 80). Duplicate samples (20 μl) of homogenate were spread onto Middlebrook 7H10 agar plates and after incubation at 37°C for 1–2 weeks the plates were examined for the presence of *M. vaccae* colonies.

TISSUE CULTURE MEDIUM

RPMI 1640 supplemented with 0·024M-sodium bicarbonate, 0·0147M-HEPES, 10 units/ml gentamicin, and foetal calf serum (FCS; Flow Laboratories, Irvine, Scotland) at 1 or 5% according to the assay, was used throughout.

SPLEEN CELL SUSPENSIONS

Three mice from each experimental group were killed by cervical dislocation and their spleens removed aseptically. The spleens were teased into tissue culture medium and a pooled single cell suspension prepared. The viability of the suspensions was usually about 80% as determined by trypan blue exclusion.

IN VITRO PROLIFERATION ASSAY

The purpose of this assay was to evaluate (^{3}H)-thymidine incorporation (lymphocyte proliferation) stimulated by a mycobacterial challenge *in vitro*. The assays were performed in 96-well, round-bottomed assay plates (Sterilin Ltd, Middlesex, England) with at least 3 wells for each experimental condition. Spleen cells (either 2×10^6 or 5×10^6 nucleated cells/ml) were cultured with heat-killed mycobacteria (10^4–10^8/ml) in a total volume of 100 μl/well. The medium was supplemented with 1% FCS. The assay plates were incubated at 37°C for 4 or 5 days in a humidified 5% CO_2-in-air atmosphere. For the last 16 h of culture 2 μl medium containing 1 μCi of (^{3}H)-thymidine (Amersham International, Amersham, England) was added. (The labelled thymidine had been previously diluted with non-radioactive thymidine to the very low specific activity of 50 mCi/mmol.) Cultures were harvested using an automatic cell harvester (Skatron, Norway) and the amount of incorporated ^{3}H determined with a Packard 'Tricarb' liquid scintillation counter (Packard Instruments Ltd, Berks., England). The background (^{3}H)-thymidine incorporation in the absence of heat-killed mycobacteria by spleen cells from immunized animals was usually greater than that from unimmunized animals (see Table 1). The data are expressed therefore in the figures as the difference between the arithmetic mean counts per minute (cpm) in the replicate wells containing spleen cells plus heat-killed organisms and similar wells without organisms (Δ cpm).

IN VITRO PRIMARY PLAQUE-FORMING CELL ASSAY

As a further measure of the immunological status of the spleen cells from the

Table 1. *In vitro* proliferative response to heat-killed *M. vaccae* of spleen cells from animals injected subcutaneously with *M. vaccae*

	Cpm of (^{3}H)-thymidine[a] incorporated into spleen cells[b] cultured with heat-killed *M. vaccae* (organisms/ml)[c]			
Spleen cells from	0	10^6	10^7	10^8
M. vaccae injected mice[d]	247 ± 15	666 ± 203 (419)[e]	730 ± 63 (483)	700 ± 106 (453)
Control mice	152 ± 15	172 ± 18 (20)	255 ± 66 (103)	242 ± 71 (90)

[a] mean ± standard deviation of replicate assays.
[b] 2×10^6 cells/ml.
[c] 5-day culture.
[d] 2×10^6 viable, 10^8 total *M. vaccae* injected subcutaneously 42 days previously.
[e] Δ cpm. Underlined values are significantly enhanced ($P < 0{\cdot}05$ or better).

immunized mice, their ability to generate primary (IgM) haemolytic plaques (1°-PFC) *in vitro* in the presence of killed mycobacteria was assessed using sheep erythrocytes (SE) as antigen. The assay was performed in Linbro 24-well, flat-bottomed assay plates (Flow Laboratories, Irvine, Scotland). 10^7 nucleated spleen cells were cultured with 4×10^5 prewashed SE (Tissue Culture Services, Berks., England) and 10^6 to 10^8 heat-killed mycobacteria in a total volume of 2 ml. The medium was supplemented with 5% FCS. The assay plates were incubated for 5 days at 37°C in 5% CO_2-in-air after which time the number of 1°-PFCs per culture (10^7 spleen cells) was determined by the method of Cunningham & Szenberg.[14] The results in the figures represent the arithmetic mean from at least 3 replicate wells.

STATISTICAL ANALYSIS

A two-tailed Student's *t* test was used to compare (a) the responses of spleen cells from the immunized animals cultured with and without heat-killed organisms, and (b) the responses of the spleen cells from immunized and unimmunized animals in the presence of the same concentration of heat-killed organisms. Our criterion for a statistically significant enhanced response due to the *in vitro* challenge was that a significant difference should be found in both comparisons. Significant responses are indicated in the table and figures ($P < 0{\cdot}05$ or better).

Results

1 SUBCUTANEOUSLY ADMINISTERED MYCOBACTERIA

Persistence of M. vaccae *in mouse tissue after injection*

No viable *M. vaccae* could be detected in the granuloma, spleen, liver or lung 35 days after the s.c. injection.

In vitro *proliferative responses of spleen cells from mice injected s.c. with* M. vaccae *or BCG*

The optimal conditions for this assay varied with factors such as the batch of CBA mice used, the time after injection and the degree of responsiveness of cells from uninjected, control mice to mycobacterial antigens (see Table 1). For this reason experiments were set up with multiple assay conditions and the results for the 'immune' spleen cells were compared with those of the control cells determined under the same assay conditions. The exact conditions used are defined in the figure legends.

Responses to the injected organism. Spleen cells from mice injected s.c. 42 days previously with *M. vaccae* incorporated more (^{3}H)-thymidine in the presence of heat-killed *M. vaccae* (HKMV) than in its absence and also when compared with

Table 2. *In vitro* proliferative response of spleen cells from mice fed *M. vaccae* in their drinking water

Spleen cells from	Cpm of (^{3}H)-thymidine[a] incorporated into spleen cells[b] cultured[c] with (organisms/ml)[c]:					
	Heat-killed *M. vaccae*			Heat-killed BCG		
	0	10^6	10^8	0	10^7	10^8
M. vaccae fed mice[d]	457 ± 111	1208 ± 172 (751)[e]	606 ± 139 (149)	364 ± 115	845 ± 258 (481)	442 ± 85 (78)
Control mice	340 ± 56	379 ± 117 (39)	724 ± 47 (384)	525 ± 227	415 ± 98 (−110)	301 ± 108 (−224)

[a] Mean ± standard deviation of triplicate assays.
[b] 5×10^6 cells/ml.
[c] 5-day culture.
[d] Mice given water contaminated with 2×10^6 viable (10^8 total) *M. vaccae*/ml per week for 3 continuous weeks, finishing 104 days previously.
[e] Δ cpm. Underlined values are significantly enhanced ($P < 0{\cdot}05$ or better).

cells from unimmunized control animals (Table 1). This enhanced proliferative response was also evident when the data were expressed as Δ cpm (Table 1). This result shows that the response was due to the *in vitro* challenge and dependent upon the prior sensitization of the cells *in vivo*, thereby indicating the presence of memory lymphocytes. The enhanced response was significant at concentrations of HKMV between 10^6 and 10^8 per ml. Similar responses could be detected with spleen cells taken from immunized mice at all times tested between 21 and 70 days after the s.c. injection although the responses were greatest between days 40 and 60. In some experiments a decline in the response to the highest concentration of HKMV was marked (data not shown). For comparative purposes the data obtained when spleen cells taken 42 days after s.c. BCG were challenged *in vitro* with various concentrations of heat-killed BCG (HKBCG) are shown (Figure 1). The responses are comparable although the decline in responsiveness to the higher concentrations of HKBCG is more dramatic in this particular experiment.

Cross-reacting responses. Mycobacterium vaccae and BCG have one group of serologically detectable antigens in common.[15] We therefore investigated how

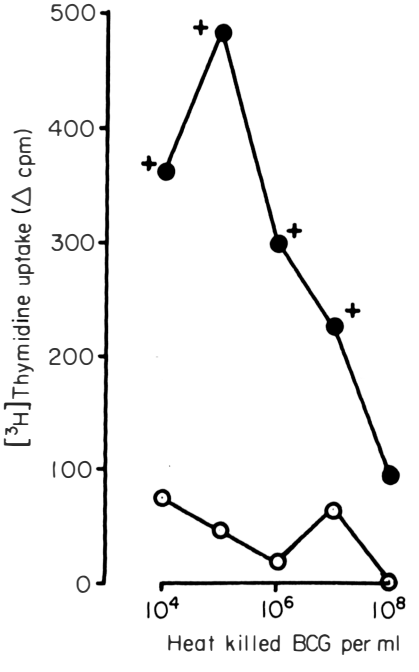

Figure 1. *In vitro* proliferative response of spleen cells to the mycobacterial species used for *in vivo* priming. Three CBA mice per group were each immunized with a single subcutaneous injection of 3×10^7 viable (6×10^7 total) BCG. Forty-two days later replicate cultures containing 2×10^5 pooled spleen cells and heat-killed BCG were set up. Culture duration was 4 days. Closed circles denote responses of spleen cells from immunized animals; open circles denote responses of uninjected control animals; + denotes statistically significantly enhanced responses.

much cross-reactivity could be detected by the sensitized splenic lymphocytes. Substantial cross-reactivity could be detected when either *M. vaccae*-sensitized spleen cells were challenged *in vitro* with HKBCG (Figure 2a) or BCG-sensitized spleen cells were challenged with HKMV (Figure 2b). Figure 2 shows the *in vitro* proliferative responses 21 days after the s.c. injection; the cross-reactivity could be detected at all time points tested between days 21 and 70 (data not shown). As with the proliferative responses to the sensitizing organism the degree of enhancement was reduced with the highest concentrations of heat-killed organism used.

Primary splenic plaque-forming cell responses in vitro

Earlier experiments have shown that the 1°-PFC response of spleen cells to SE *in vitro* is enhanced in the presence of added HKBCG when the mice have been

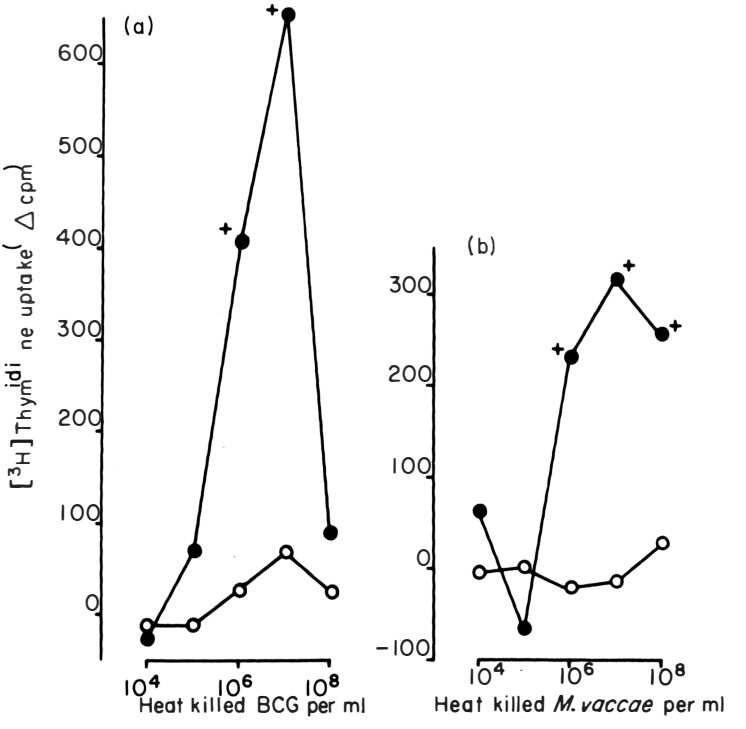

Figure 2. *In vitro* proliferative cross-reacting response of spleen cells to the alternative mycobacterial species. Three CBA mice per group were each immunized with a single subcutaneous injection of (a) 2×10^6 viable (10^8 total) *M. vaccae* or (b) 3×10^7 viable (6×10^7 total) BCG. Twenty-one days later replicate cultures containing 2×10^5 pooled spleen cells and either (a) heat-killed BCG or (b) heat-killed *M. vaccae* were set up. Culture duration was (a) 5 days and (b) 4 days. Closed circles denote responses of spleen cells from immunized animals; open circles denote responses of cells from uninjected control animals; + denotes statistically significantly enhanced responses.

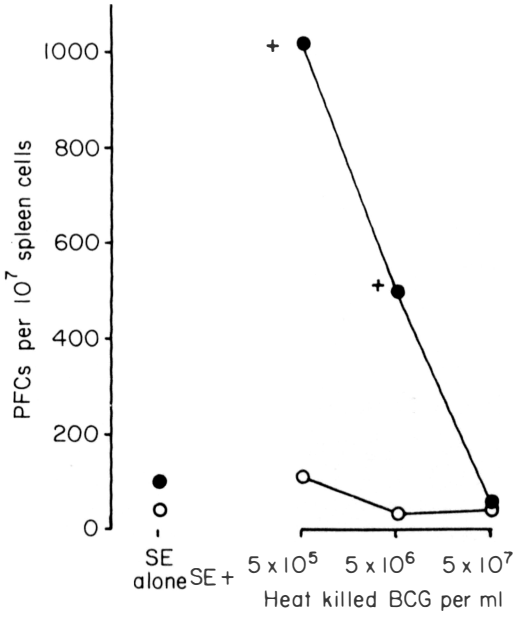

Figure 3. Enhancement of the number of primary plaque-forming cells (1°-PFCs) to sheep erythrocytes (SE) in *M. vaccae*-sensitized spleen cell suspensions challenged *in vitro* with HKBCG. Three CBA mice per group were each immunized with a single subcutaneous injection of 2×10^6 viable (10^8 total) *M. vaccae*. Forty-two days later replicate cultures containing 1×10^7 pooled spleen cells were incubated with 4×10^5 SE, alone or with heat-killed BCG for 5 days. Values shown are the mean numbers of 1°-PFCs per 10^7 spleen cells taken from immunized animals (closed circles) or uninjected control animals (open circles). + denotes statistically significantly enhanced responses.

previously injected with BCG intravenously[13] or s.c. (Swinburne, Brown and Brown; manuscript in preparation). In this study we investigated whether such an enhancement occurred after s.c. injection of *M. vaccae*. When cultured *in vitro* with HKBCG, spleen cells from mice injected s.c. 42 days previously with *M. vaccae* gave markedly enhanced 1°-PFC responses when compared with cells from uninjected control animals (Figure 3). The enhancement was greatest with the lowest concentration of HKBCG used (5×10^5/ml) and declined as the concentration was increased to 5×10^7/ml. As with the proliferative responses this enhancement was dependent upon the prior sensitization of the spleen cells *in vivo* and the presence of HKBCG *in vitro* demonstrating an anamnestic response to common antigens. Similar responses were detected if HKMV was used *in vitro* and responses to both species could be detected throughout the experimental period (21–70 days after injection—data not shown).

2 ORALLY ADMINISTERED MYCOBACTERIA

Persistence of M. vaccae *in mouse tissues after feeding*

When the spleen, liver and lungs of the mice fed with *M. vaccae* were examined

104 days after the feeding was stopped no viable *M. vaccae* were detected in any of the tissues.

In vitro *proliferative responses of spleen cells from mice fed* M. vaccae

Spleen cells from mice fed *M. vaccae* 104 days previously and incubated with 10^6 HKMV/ml gave significantly enhanced proliferative responses both above background (no HKMV) and compared with cells from control mice incubated with 10^6 HKMV/ml (Table 2). A similar response was elicited using 10^7 HKBCG/ml. Cells from the control mice gave a significantly enhanced response to 10^8 HKMV/ml compared to background (^{3}H)-thymidine incorporation (no HKMV). This was presumably a result of exposure of the mice to mycobacterial antigens before their isolation and has been a common observation with cells from our experimental animals. The response to HKMV by such cells is invariably higher than that to HKBCG: Table 2 shows the same cell suspension gave no response to HKBCG.

Discussion

These experiments show that this strain of *M. vaccae* isolated from a BCG vaccination trial area (in Uganda) can generate an immune response in mice comparable to that of Glaxo BCG, after either subcutaneous injection or oral administration of the bacilli, as detected by the presence of memory lymphocytes in the spleen. The anamnestic response to the subcutaneous injection was detected using two separate assays, both of which involve macrophage–T lymphocyte interactions with the mycobacterial antigens. The overall magnitude of the response was influenced by the quantity of heat-killed organisms (antigen) used *in vitro*. Similar results were obtained when the mice were given *M. vaccae* orally although only the proliferation assay was used in these experiments. It should be emphasized that the low levels of (^{3}H)-thymidine incorporated by the proliferating spleen cells reflect the low specific activity of (^{3}H)-thymidine label used and not a poor proliferative response. This low specific activity was chosen to minimize possible fluctuations in (^{3}H)-thymidine incorporation due to the well-documented synthesis and release of non-radioactive thymidine by the macrophages in the cultures.[16]

Other workers using a different strain of *M. vaccae* have reported that the *M. vaccae* sensitizes guinea-pigs and mice only poorly as measured by a delayed footpad swelling response to cytoplasmic extracts of *M. vaccae* or whole bacilli.[10,17] Good responses were obtained only if the *M. vaccae* was incorporated in Freund's adjuvant.[17] Interestingly, although Shepard, van Landingham & Walker[11] were unable to detect a delayed-type hypersensitivity (DTH) response in mice sensitized with *M. vaccae* and challenged with a soluble extract of *M. leprae*,

these mice did show both a swelling of the lymph node draining the sensitization site and also a limited protective immunity against viable *M. leprae*. Their findings are consistent with the observations of others that DTH responses are dissociable from protective immunity.[18,19] Using the same strain of *M. vaccae* as used here, Rook & Stanford[20] detected good footpad swelling responses to vaccin in BALB/c mice sensitized s.c. with live *M. vaccae*. This difference in results reported by the various groups may reflect strain differences between the *M. vaccae* isolates which were demonstrated to exist by Watson *et al.*[17]

In immunoprecipitation tests using rabbit antisera and sonicates of bacilli, *M. vaccae* and BCG appear to share only the common (group 1) mycobacterial antigens.[15] Cross-reactions detected in our study may therefore be due to antigens common to all mycobacteria. (The medium on which the mycobacteria were grown is non-antigenic.) The cross-reactivity is particularly relevant as evidence is now accumulating that certain epitopes on the common mycobacterial antigens are responsible for evoking a protective immune response in the host.[3, 15, 21]

M. vaccae has been considered as a potential candidate for a leprosy vaccine.[9, 11, 17, 22] However, in animal models, *M. vaccae* has ranked a poor third behind *M. leprae* itself and BCG.[11] This ranking may be due to selection of an inappropriate strain of *M. vaccae*. Further, the incentive to pursue studies on *M. vaccae* has been reduced by the observation that other species of mycobacteria need to persist in the tissues to induce protection against pathogenic mycobacteria in mice.[12] It has subsequently been assumed, but not proven, that the same requirement would be necessary for *M. vaccae*, a species reported to persist very poorly.[10] In our experiments the *M. vaccae* was observed not to persist *in vivo* although the presence of memory lymphocytes in the spleen was detected 104 days after feeding with *M. vaccae* had stopped. We have also detected memory lymphocytes at earlier times when no *M. vaccae* was detected in the tissues (data not shown). This indicated that persistence of viable *M. vaccae* was not a requirement for the development or persistence of such memory lymphocytes. Whether or not these are T lymphocytes that also recognize *M. leprae* antigens is currently being investigated.

The observation that *M. vaccae* can sensitize mice as effectively as Glaxo BCG supports the view that the apparent failure of BCG vaccination against leprosy in certain areas may be because the population is already sensitized. The relationship of those responses to protective immunity is currently being studied.

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