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## Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy

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P. E. M. FINE<sup>1\*</sup>, S. FLOYD<sup>1</sup>, J. L. STANFORD<sup>2</sup>, P. NKHOSA<sup>3</sup>, A. KASUNGA<sup>3</sup>,  
S. CHAGULUKA<sup>3</sup>, D. K. WARNDORFF<sup>1,3</sup>, P. A. JENKINS<sup>4</sup>, M. YATES<sup>5</sup>  
AND J. M. PONNIGHAUS<sup>3,6</sup>

<sup>1</sup> Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT

<sup>2</sup> Department of Medical Microbiology, Royal Free and University College Medical School, Windeyer Institute of Medical Sciences, 46 Cleveland Street, London W1P 6DB

<sup>3</sup> Karonga Prevention Study, PO Box 46, Chilumba, Karonga District, Malawi

<sup>4</sup> Bryn Glas, Llangcitho, nr Tregaron, Cardiganshire SY25 6TR, UK

<sup>5</sup> Mycobacterial Reference Laboratory, Kings College Hospital (Dulwich), East Dulwich Grove, London SE22 8QF

<sup>6</sup> Pappelweg 08548 Syrau Germany

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### SUMMARY

More than 36000 individuals living in rural Malawi were skin tested with antigens derived from 12 different species of environmental mycobacteria. Most were simultaneously tested with RT23 tuberculin, and all were followed up for both tuberculosis and leprosy incidence. Skin test results indicated widespread sensitivity to the environmental antigens, in particular to *Mycobacterium scrofulaceum*, *M. intracellulare* and one strain of *M. fortuitum*. Individuals with evidence of exposure to ‘fast growers’ (i.e. with induration to antigens from fast growers which exceeded their sensitivity to tuberculin), but not those exposed to ‘slow growers’, were at reduced risk of contracting both tuberculosis and leprosy, compared to individuals whose indurations to the environmental antigen were less than that to tuberculin. This evidence for cross protection from natural exposure to certain environmental mycobacteria may explain geographic distributions of mycobacterial disease and has important implications for the mechanisms and measurement of protection by mycobacterial vaccines.

### INTRODUCTION

Of more than 100 known species of the genus *Mycobacterium*, the large majority are described as ‘environmental’ (or ‘atypical’) mycobacteria, and are thought to live as saprophytes in water or soil. Though these environmental mycobacteria infrequently cause disease (in contrast to the obligate parasites *M. tuberculosis* and *M. leprae*), they are of importance for human health for at least four reasons.

\* Author for correspondence.

First, several species have long been known to cause disease under special circumstances, and are hence known as ‘opportunistic’ mycobacteria. Such opportunities include their presentation in aerosols, allowing them to reach the peripheral lung fields, and also immunological inadequacy, due to immaturity as in cervical lymphadenitis of childhood [1], or due to immunosuppression induced by high dose corticosteroids or by chronic virus infections such as HIV. Thus *M. avium* has been a major cause of mortality in AIDS patients in Europe and North America [2].

Second, two species are associated predominantly with skin lesions: *M. marinum* causes minor granulomatous lesions, and *M. ulcerans* is responsible for a severe necrotizing disease, Buruli ulcer, which has replaced leprosy as the second most common mycobacterial disease, after tuberculosis, in parts of West Africa [3]. Third, these organisms are ubiquitous in the environment, including piped water supplies, and commonly contaminate sputum smears and cultures, leading to false positive diagnoses of tuberculosis, when these are based upon sputum smear alone [4]. Finally, there is considerable evidence that exposure of human populations to these organisms can have immunological effects which may on the one hand protect against tuberculosis or leprosy (and hence be important in determining the geographic distribution of these diseases), and on the other hand may mask protection by BCG vaccines [5]. Besides these BCG-related effects, contact with environmental mycobacteria probably plays an important role in training the infant immune system, thereby establishing the pattern of immunological response to other bacterial, and perhaps viral, infections, to allergen challenges and to surveillance for malignant cells. In addition to these direct implications for human health, mycobacteria are important and ubiquitous components of the normal soil flora, and doubtless play a major ecological role in the biosphere.

Despite their importance, the environmental mycobacteria are little studied and not well understood. This ignorance is in part a consequence of the difficulties in working with them: they grow relatively slowly compared to other bacteria, most require special decontamination methods to separate them from the many other microbes which share their environments, and some have never been grown *in vitro* at all [6]. Modern methods of identification based upon DNA probes may facilitate efforts to survey the environment, but the traditional methods of culture and skin testing have provided most information to date. The development of specific skin test reagents for various environmental mycobacteria, and their use to survey human populations, was initiated in the 1960s in major studies aimed at understanding geographic variations in apparent protection imparted by BCG vaccines [7, 8]. These studies have shown that human populations in many areas of the world have been exposed and sensitized to antigens of environmental mycobacteria, and that this exposure may be particularly intense in the tropical regions. This geographic association is part of the argument for

their role in determining the low efficacy of BCG in adults observed in many tropical populations [9].

We here present the results of studies to describe the environmental mycobacterial flora in a rural district of northern Malawi, based upon both skin testing and the isolation and identification of contaminants in sputum cultures. The work took place within the context of a large epidemiological study of leprosy and tuberculosis, and provided an opportunity to test directly the hypothesis that prior exposure to environmental mycobacteria influences the risks of these important mycobacterial diseases.

## MATERIALS AND METHODS

The work described here was carried out within what has become known as the Karonga Prevention Study, the basic methods of which have been described in detail elsewhere [10, 11]. In brief, the skin testing presented in this paper was performed within a total population survey of the Karonga District population carried out during 1980–5 ('LEP-1'). Teams of trained interviewers and paramedical workers moved systematically across the district, interviewing and examining all individuals on a household-by-household basis. The examinations included inspection for a BCG scar and application of one or two skin tests, usually RT23 tuberculin in one arm, and an environmental mycobacterial antigen in the other arm. The environmental antigens were applied in succession, so that all individuals in a given area were tested with a certain specific environmental antigen, along with either RT23 tuberculin or a second environmental antigen.

### Antigens

RT23 tuberculin (2 IU) was purchased from the Danish Statens Serum Institute. Fifteen different environmental mycobacterial antigens were prepared (by J.L.S.) from both slow growers (*M. avium* (2 strains), *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. xenopi*) and fast growers (*M. chitae*, *M. fortuitum* (3 strains), *M. neoaurum*, *M. nonchromogenicum*, *M. vaccae*). Two of the *fortuitum* antigens (serotypes 1 and 2-3-4) were derived from autochthonous strains isolated (by P.A.J.) from soil samples collected in Karonga District. All were prepared by sonication in borate-buffered saline of organisms grown on Sauton's medium and sterilized by filtration. The reagents were

standardized to a concentration equivalent to 2 TU of RT23 [12].

### Skin testing procedure

Skin tests were carried out on the volar surface of the arm. All tests involved intradermal injection of 0.1 ml of reagent, with a sterile 26 gauge needle. Tests were read at 48–72 h, with induration diameters measured along and across the arm. Average diameters are used in the analyses presented here.

### Tuberculosis and leprosy

Data on tuberculosis and leprosy incidence were collected by active follow-up of the population within a second total population survey carried out during 1986–9 ('LEP-2') [13]. Diagnostic methods (tuberculosis bacteriology and leprosy biopsies) are described in detail elsewhere [14, 15].

### Mycobacterial isolates

In addition to skin test results, we present data on environmental mycobacteria isolated from sputum cultures (acid egg medium with and without pyruvate) of tuberculosis suspects, carried out during 1985–98. In general, only colonies which looked macroscopically as though they might harbour *M. tuberculosis* were sent for species identification (to P.A.J. until 1996 and to M.Y. thereafter).

### Data handling and statistical analyses

All data were coded at project headquarters in Malawi, entered on computers and accumulated on databases at the London School of Hygiene and Tropical Medicine in London. Analyses presented here were performed in STATA, using logistic and Poisson regression. In order to examine the association of prior sensitivity to mycobacteria with tuberculosis and leprosy, we have compared pooled data on slow growers with those from fast growers, as there were insufficient cases among those tested with single antigens. Because of evidence for cross-reactivity between antigens, we assume that individuals with skin test induration to antigen X which is greater than their induration to RT23 tuberculin are more likely to have been infected with *Mycobacterium* X than are those whose induration to antigen X is less than that to tuberculin. Cases of TB and leprosy that

arose within 6 months of skin-testing, and individuals whose tuberculin test and environmental mycobacterial test were read more than 6 h apart, were excluded from the incidence rate analyses.

## RESULTS

Results are available from 31044 individuals dual-tested with both an environmental mycobacterial antigen and RT23 tuberculin, and from 5146 individuals for whom the second test was not with RT23. Table 1 shows the numbers of tests carried out with each of the antigens, during the LEP-1 survey. Frequency distributions of induration sizes for each antigen are presented in Fig. 1. We see that, for each of these antigens, some individuals developed no apparent response at all, and others developed indurations of roughly 5–15 mm, with a mode near 10 mm. Though the illustrated distributions are for all ages combined, the age, sex and BCG scar status distributions of the separate tested populations are sufficiently similar for them to be compared. In addition, Table 1 shows, for each antigen, the prevalence of sensitivity (defined as induration  $\geq 5$  mm, column 3; or  $\geq 10$  mm, column 4), standardized for age, sex and BCG-scar status. Using these criteria, the prevalence of sensitivity is seen to vary greatly between the different mycobacterial reagents, with *M. intracellulare* and *M. scrofulaceum* the highest, and *M. xenopi* the lowest.

For each antigen, the proportion of individuals with sensitivity greater than any given criterion increased with age. For most of the antigens these proportions were significantly higher among individuals with than among those without BCG scars. This BCG effect was particularly strong for the slow growers in general, and least convincing for *M. fortuitum*, *M. gordonae* and *M. nonchromogenicum*. The proportion with induration  $\geq 5$  mm was similar for male and female children, but higher among males than among females for individuals above 15 years of age except for *M. chitae*, *M. fortuitum*, *M. gordonae* and *M. xenopi*. These trends are illustrated in Fig. 2 for *M. scrofulaceum* and *M. neoaurum*. For each antigen, the significance of the interaction between age and sex is indicated in column 7, and the significance of the BCG effect is indicated in column 9, of Table 1.

The last two columns in Table 1 present results for those environmental mycobacterial reagent tests which were paired with RT23 tuberculin, and are here

Table 1. Summary of data on environmental mycobacterial skin tests carried out in Karonga district, northern Malawi, 1980–4. Prevalences (columns 3 and 4) refer to individuals greater than 20 years of age, and are age–sex–BCG standardized against the total RT23 population. The last two columns are restricted to tests paired with RT23 tuberculin, and adjusted for RT23 induration

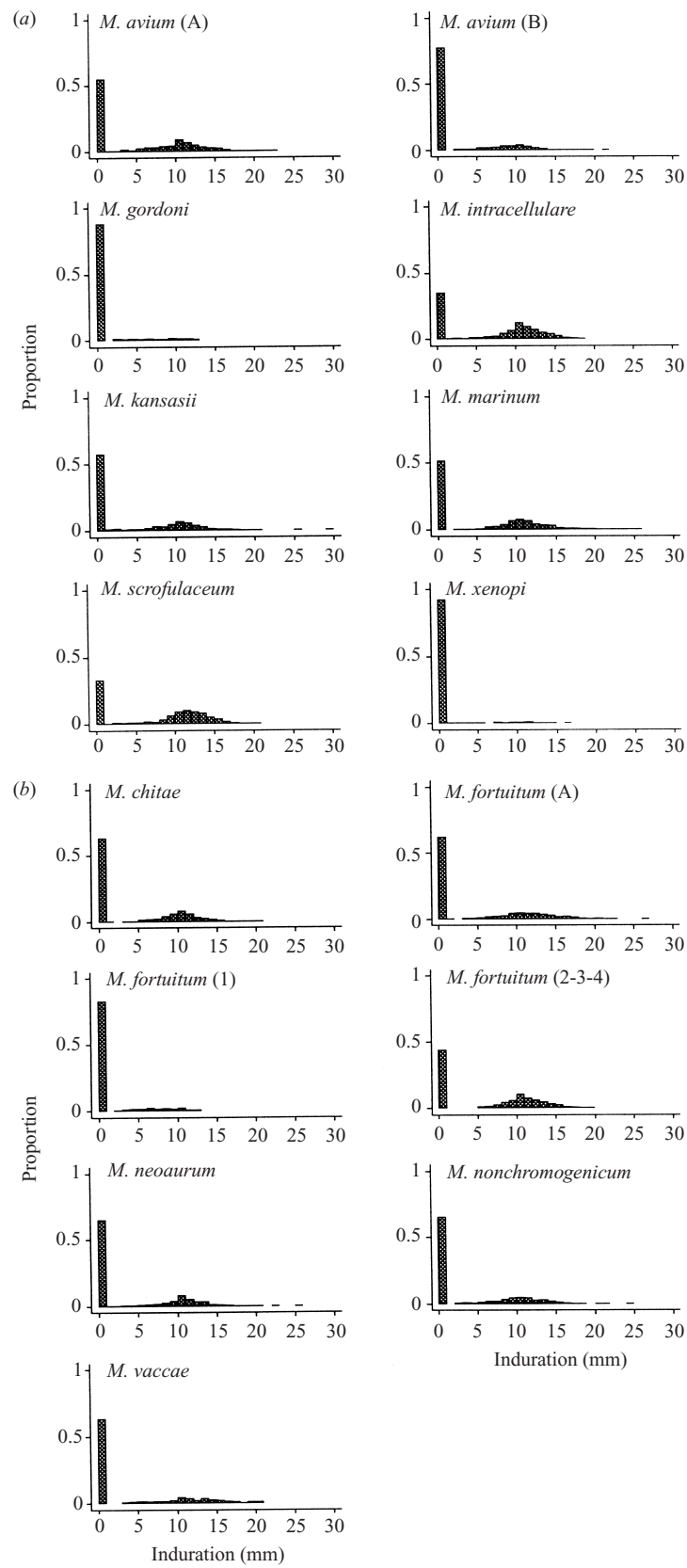
Mycobacterium	n	≥ 5 mm (%)	≥ 10 mm (%)	OR, female to male* (95% C.I.)		Age by sex interaction P value†	OR, BCG positive to BCG negative (95% C.I.)	BCG effect P value	Adjusted for RT23 induration in paired test‡	
				< 15 years	≥ 15 years				Deviance change when include RT23§	Age-sex interaction (P value)
Slow growers										
<i>M. avium</i> (A)	2623	58	42	1.02 (0.79, 1.32)	0.68 (0.54, 0.85)	0.019	1.17 (0.97, 1.42)	0.101	0.216	0.058
<i>M. avium</i> (B)	1699	32	18	0.93 (0.59, 1.46)	0.57 (0.42, 0.76)	0.074	1.74 (1.27, 2.38)	< 0.001	0.222	0.206
<i>M. goodii</i>	2014	14	7	0.63 (0.41, 0.99)	0.55 (0.40, 0.77)	0.635	1.28 (0.93, 1.75)	0.123	0.120	0.184
<i>M. intracellulare</i>	2886	77	60	1.15 (0.93, 1.42)	0.67 (0.50, 0.90)	0.003	1.39 (1.15, 1.68)	< 0.001	0.154	0.157
<i>M. kansasii</i>	2393	59	44	0.97 (0.72, 1.29)	0.41 (0.32, 0.53)	< 0.001	1.80 (1.46, 2.23)	< 0.001	0.276	< 0.001
<i>M. marinum</i>	3020	59	44	1.04 (0.84, 1.29)	0.59 (0.48, 0.73)	< 0.001	1.81 (1.51, 2.18)	< 0.001	0.315	0.044
<i>M. scrofulaceum</i>	3324	77	66	1.15 (0.95, 1.40)	0.48 (0.37, 0.62)	< 0.001	1.55 (1.30, 1.86)	< 0.001	0.157	< 0.001
<i>M. xenopi</i>	3010	11	6	0.20 (0.09, 0.45)	0.38 (0.28, 0.50)	0.125	1.54 (1.15, 2.07)	0.004	0.064	0.854
All slow growers	20969	48	36							
Fast growers										
<i>M. chitae</i>	3232	47	29	0.98 (0.78, 1.23)	0.83 (0.68, 1.01)	0.283	1.27 (1.06, 1.51)	0.008	0.125	0.914
<i>M. fortuitum</i> (A)	1744	44	31	1.01 (0.75, 1.37)	0.88 (0.67, 1.15)	0.492	1.07 (0.85, 1.35)	0.560	0.108	0.880
<i>M. fortuitum</i> (1)	1139	24	8	1.23 (0.60, 2.56)	0.52 (0.34, 0.78)	0.040	1.26 (0.81, 1.96)	0.309	0.136	0.050
<i>M. fortuitum</i> (2-3-4)	3271	71	53	0.82 (0.67, 1.01)	0.89 (0.71, 1.11)	0.624	0.95 (0.80, 1.12)	0.515	0.086	0.574
<i>M. neoaurum</i>	3465	43	31	1.26 (0.99, 1.60)	0.64 (0.53, 0.78)	< 0.001	1.29 (1.08, 1.55)	0.005	0.145	0.001
<i>M. nonchromogenicum</i>	1488	50	34	0.97 (0.64, 1.46)	0.42 (0.31, 0.56)	0.001	1.15 (0.86, 1.55)	0.348	0.151	0.020
<i>M. vaccae</i>	882	43	33	1.09 (0.71, 1.66)	0.54 (0.37, 0.80)	0.018	1.39 (0.99, 1.96)	0.059	0.105	0.035
All fast growers	15221	46	31							
RT23	48128	59	48	0.92 (0.86, 0.98)	0.61 (0.58, 0.65)	< 0.001	1.90 (1.81, 1.99)	< 0.001		

\* OR is for the odds of having an induration of ≥ 5 mm.

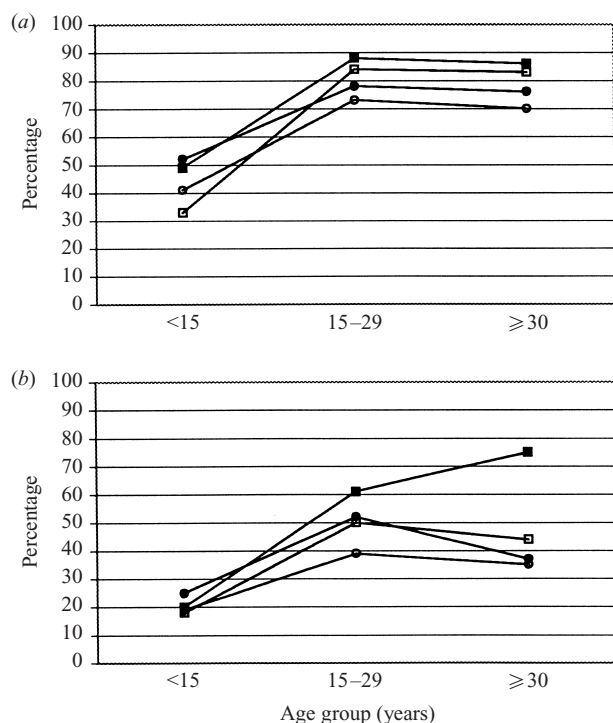
† test for whether female to male odds ratio of having an induration of ≥ 5 mm is different for those aged less than 15 years compared to those aged 15 or over.

‡ RT23 induration was categorised as 0 mm, 1–5 mm, 6–10 mm, 11–15 mm, > 15 mm.

§ (change in model deviance when include RT23 induration as a predictor of NTM induration)/(number of observations in data set). Reflects strength of association between RT23 induration and NTM induration, standardized for size of data set (all associations  $P < 0.0001$ ).



**Fig. 1.** Frequency distributions of skin test indurations to environmental mycobacterial antigens, Karonga District, northern Malawi, 1980–5: (a) slow growers; (b) fast growers.



**Fig. 2.** Percentage with skin test induration  $\geq$  mm for (a) *M. scrofulaceum* and (b) *M. neoaurum* antigens, by age group, sex and BCG scar status: Karonga District, northern Malawi, 1980–5. ■, M BCG +ve; □, M BCG –ve; ●, F BCG +ve; ○, F BCG –ve.

**Table 2.** *Environmental mycobacteria species (or species groups) identified in sputum samples collected in Karonga District, northern Malawi, 1985–98*

Slow growers		Fast growers	
Species (group)	<i>n</i>	Species (group)	<i>n</i>
<i>M. avium-intracellulare</i>	249	<i>M. fortuitum</i>	117
<i>M. gordonae</i>	41	<i>M. chelonae</i>	8
<i>M. terrae</i>	30	<i>M. flavescens</i>	5
<i>M. kansasii</i>	17	<i>M. smegmatis-phlei</i>	5
<i>M. malmoense</i>	2		
Total	339	Total	135

adjusted for the RT23 tuberculin induration. Column 10 provides a measure of the strength of the association between the induration to an environmental mycobacterial reagent and the induration to RT23, and column 11 shows the age–sex interaction after controlling for RT23 induration as a predictor of induration to the environmental mycobacterial antigen.

Table 2 shows the numbers of isolates of different environmental mycobacteria identified in sputum

cultures collected from tuberculosis suspects between 1985 and 1998. Members of the *M. avium–intracellulare* complex were most commonly found, followed by *M. fortuitum*. Interestingly, the relative frequency of these species corresponds to the relative prevalence of sensitivity to their antigens, as shown in Table 1 (columns 3 and 4). Several other species were identified less commonly in these cultures.

The implications of prior sensitivity to slow or to fast growing environmental mycobacteria for subsequent incidence of tuberculosis and leprosy are explored in Table 3. These are expressed as rate ratios (with 95% CI), comparing the incidence among individuals whose prior sensitivity to the environmental mycobacterial antigen was greater than that to RT23, to the incidence among individuals whose sensitivity to the environmental mycobacterial antigen was less than or equal to that to RT23. We see evidence ( $P < 0.10$  for TB and  $P = 0.05$  for leprosy) that prior sensitivity to antigens of fast growers, but not to antigens of slow growers, was associated with low risks of both leprosy and tuberculosis.

## DISCUSSION

These data show that a variety of different environmental mycobacteria are present in Karonga District, and that many people living there have been sensitized by or to their antigens. Nine different ‘species’ (or species groups) have been identified to date in sputum cultures, as shown in Table 2. We do not know the actual source of these organisms. It is likely that several of them had colonized or infected the individuals who provided the sputum samples, but others probably arose through contamination of the sputa either in the field, or in the laboratory. We have been unable to identify evidence of contamination in the laboratory, in terms of clusters of specimens with the same environmental mycobacterium processed on the same day, but nonetheless expect that some of the isolates originated in this way. We note that more slow-growing than fast-growing organisms were identified in the sputa. This should be interpreted with caution, however, since fast-growers tend to be more susceptible to sputum decontamination techniques than are slow growers, and sputum cultures were in general only sent to the reference laboratory for species confirmation if the primary isolates looked as though they might be *M. tuberculosis* (16% of cultures grew something which was ultimately sent to a reference laboratory). Some mycobacteria such as

Table 3. Implications of prior sensitivity to environmental mycobacteria for subsequent incidence rates of tuberculosis and leprosy, Karonga District, northern Malawi, 1980–90

	Tuberculosis			Leprosy		
	t/pyears*	rate ratio† (95% CI)	P value‡	l/pyears*	rate ratio§ (95% CI)	P value‡
Slow growers						
NTM ≤ RT23	15/65869			51/50892		
NTM > RT23	4/19608	1.08 (0.34, 3.43)	0.899	14/14787	1.09 (0.59, 2.02)	0.788
Fast growers						
NTM ≤ RT23	26/542352			30/41044		
NTM > RT23	2/15213	0.32 (0.07, 1.39)	0.080	2/11488	0.29 (0.07, 1.25)	0.050

Incidence rate ratios (with 95% confidence intervals) compare the incidence among individuals whose prior sensitivity to the environmental mycobacterial antigen was greater than that to RT23 tuberculin (NTM > RT23), to the incidence among individuals whose sensitivity to the environmental mycobacterial antigen was (less than or equal to) that to RT23 (NTM ≤ RT23).

\* t/pyears TB cases/person-years; l/pyears, leprosy cases/person-years.

† Adjusted for age, sex, BCG scar status, and RT23 induration; RT23 induration categorised as 0 mm, 1–5 mm, 6–10 mm, 11–15 mm, > 15 mm.

‡ P value is for likelihood ratio test; Wald test, to which (approximate) 95% CI relate, gives slightly higher P values

§ Adjusted for age, sex, BCG scar status, zone and RT23 induration; RT23 induration categorized as 0 mm, 1–5 mm, 6–10 mm, 11–15 mm, > 15 mm.

*M. gordonae*, *M. scrofulaceum* and *M. neoaurum* can produce bright yellow colonies which do not look like those of tubercle bacilli, and are thus less likely to have been submitted for investigation in this study. A special investigation is now underway to identify where in the environment these various organisms normally live.

In addition to the direct evidence of these isolated organisms, we have evidence of widespread skin test sensitivity to antigens of various environmental mycobacteria (Table 1, Figs 1 and 2). Though the frequency distributions of skin test induration to each of the antigens look bimodal (some individuals with no sensitivity, and some with a range of indurations, typically around 10 mm), we wish to avoid a simplistic interpretation of test “positivity”. The significant association between sensitivity to most of the antigens (as defined by induration of at least 5 or 10 mm) with the presence of a BCG scar indicates that the delayed-type hypersensitivity should not be interpreted as specific evidence of exposure to that organism. This is not surprising, as it is well known that members of the genus *Mycobacterium* share many antigens in common. This cross-reactivity is also evident in these analyses in the association of sensitivity between the various antigens and RT23, which was very strong, and particularly the fact that the strength of this association increased with phylogenetic closeness to *M. tuberculosis*. Thus evidence of association was strongest for *M. marinum* and *M. kansasii*, followed

by *M. avium*, *M. scrofulaceum* and *M. intracellulare*. There is also evidence for antigen specificity in these data, in the different prevalences of the various antigens, and the fact that the high relative prevalence of sensitivity to *M. avium* (*A*), *M. intracellulare* and *M. fortuitum* (2-3-4) correspond to their relative frequencies of these species among sputum contaminants (see Tables 1, 2). In this context, the relatively low prevalence of skin test responses to the *M. gordonae* reagent, in relation to the culture isolation figures, may be due to variation within this species, and the fact that the skin test reagent was prepared from the type strain originating from Central America.

We find that prevalence of sensitivity to all of the antigens is similar among male and female children (< 15 years of age) but that for most (9/15) of the antigens the prevalence of sensitivity is significantly higher among males for adults. This interaction remains significant for 6 out of the 12 environmental mycobacteria (and to antigens from one strain of *M. fortuitum*) after adjustment for RT23 sensitivity (Table 1). Such an age–sex interaction has long been known for tuberculin [16], and has been reported for *M. intracellulare* [17]. There are three potential explanations for this pattern. First, it may indicate that males are consistently more exposed to these antigens than are females. This hypothesis is difficult to test, but it does not fit obviously with our observations of the Karonga population. Both males



and females work in agricultural fields, and females have much more contact than males to water sources such as streams and boreholes, which are often surrounded by muddy areas. Second, it may reflect shared antigenicity with the tubercle bacillus, and hence be a reflection of greater exposure of males to tubercle bacilli, (if indeed the higher prevalence of sensitivity to tuberculin reflects exposure to infection with *M. tuberculosis*). The fact that the age-sex interaction remains after adjustment for RT23 for half the environmental mycobacteria tested is evidence against this hypothesis. Thirdly, it may reflect a greater propensity for males, compared to females, to develop a delayed type hypersensitivity response to mycobacterial antigens. We favour the latter hypothesis, noting its corollary, that the greater sensitivity to tuberculin among adult males compared to adult females is also at least in part a reflection of this physiological difference. Such a physiological difference has been suggested by other workers [6, 18]. It is potentially important for our general understanding of tuberculosis, as it implies a sex difference in the sensitivity and specificity of tuberculin reactivity in humans, which has not been appreciated heretofore.

It should be noted that these data were collected during the early and mid 1980s, when the prevalence of HIV was less than 1% in this population. The patterns observed were thus not a reflection of HIV-attributable immunosuppression.

The indication in these data of reduced risks of both leprosy and tuberculosis associated with prior sensitivity to fast-growing, but not to slow-growing environmental mycobacteria is particularly intriguing. The associations ( $P = 0.05$  for leprosy and  $P < 0.1$  for tuberculosis) are present after adjustment for RT23 sensitivity, and are thus not merely a reflection of relatively low RT23 sensitivity in individuals with greater induration to the environmental mycobacteria – let alone the fact that low RT23 sensitivity is associated with low tuberculosis incidence but high leprosy incidence [19]. That sensitivity to antigens of fast but not of slow growers should be associated with reduced risks of both diseases was contrary to the initial hypothesis of some of the authors, who had expected that natural protection would be more likely from exposure to the more closely related slow-growing organisms (the closest precedent for such an analysis is in data from US naval recruits suggesting low tuberculosis incidence associated with sensitivity to antigens of *M. intracellulare*, a slow grower [20]). The argument by

genetic proximity has been countered by others among us who have argued that fast-growing mycobacteria tend to be better adjuvants for protective immune responses than are slow growers [21, 22]. The rate ratios observed here appear to support the latter hypothesis. If delayed-type hypersensitivity to either the tubercle or leprosy bacilli were not protective, but adversely pre-sensitizing, perhaps via TH2 adjuvant activity, this may predispose to an adverse clinical outcome if an individual is later exposed to and infected by the pathogen [23]. Such an explanation would fit with several lines of evidence indicating the dissociation of DTH and of protective immunity [19, 24].

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