Isolation of environment-derived *Mycobacterium leprae* from soil in Bombay

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It is well known that even in highly endemic areas, contact with a leprosy patient cannot be established as a source of infection in a considerable proportion of the new cases.¹¹ In a study covering Hawaii, Philippines, Indonesia and some countries in Africa, no contact could be established in 30–60% of the new patients.¹ Enna *et al.*³ reported that in the continental United States only 25·8% of new leprosy cases had any known contact with leprosy patients.

Accordingly, attention is drawn to the possible existence of extra-human reservoirs and thus to extra-human sources of infection.¹⁵ At the II International Leprosy Congress in 1909, Sand¹⁷ postulated, on the basis of epidemiological observations, that leprosy is not transmitted by direct contact, but probably through some environmental medium such as soil. The finding in the 1970's of naturally acquired leprosy in the nine-banded armadillo¹³ and in the Mangabey monkey¹⁸ may be consistent with this hypothesis.

At the same time, evidence has accumulated supporting the potential existence of environmental sources of *M. leprae*, particularly in sphagnum vegetation.^{8,5,14,4} However, until recently, the lack of sufficiently specific methods has hindered a thorough differentiation of the mycobacteria found in series of samples, obtained from various environmental sources.

In the present paper, a technique based on monoclonal antibodies specific for the major *M. leprae* phenolic glycolipid¹⁹ was used, in addition to already established procedures, for further differentiation of the mycobacterial content of the samples.

Materials and methods

A total of 17 samples, 13 water samples and four soil samples, was randomly

selected from six districts of the Bombay Leprosy Project area and in Kopary leprosy colony (Table 1), covering approximately 1 million of greater Bombay's total population of 8 million (Figure 1). In this area, about 40% of the population is living in slums.

The water samples were filtered with Antlia pneumatic pump SP 050/2 through membrane filter pore size $0.5\ \mu\text{m}$ (Schleicher and Schüll, Dassel). The filters were kept in a refrigerator at $1^{\circ}\text{--}4^{\circ}\text{C}$ until processing. The soil samples were collected with sterile spoons into sterile plastic bags and refrigerated until processing. The membrane filters were homogenized in 3 ml of 0.1% sterile albumin solution, the soil samples were homogenized with equal volume of sterile distilled water allowed to stand for 10 min, the supernatant centri. 30 min at 5000 upm and resuspended in 3 ml of 0.1% albumin solution. From the fluids smears were prepared for counting of acid-fast bacilli.¹⁰

One millilitre of the fluid was treated with oxalic acid and NaOH^2 and inoculated on Löwenstein-Jensen, Middlebrook 7 H10 solid media in tubes and Middlebrook 7 H10 medium in plates. The plates were incubated for 6 weeks and the tubes 4 months at 31°C . The differentiation and taxonomic analysis of isolated mycobacteria were based on 46 properties.⁷ Another portion of the fluid was inoculated, without treatment, subcutaneously (0.03 ml) into both hind footpads of 10 female white mice of the inbred strain NMRI-SPF (Lippische Versuchstierzucht, Extertal, W. Germany). The examination for acid-fast bacilli (AFB) was made at intervals of 6, 9, 12, 18 and 24 months.¹⁰ AFB-positive homogenates from the footpads were examined for cultivable mycobacteria, and for further maintenance inoculated in sphagnum nutritive substrate.⁹ When cultivable mycobacteria were excluded, 0.03 ml from the sphagnum substrate was inoculated in nu/nu mouse footpads (Gl. Bomholtgard, Ry, Denmark). From each sample at least 5 nu/nu mice were inoculated. The footpads were examined after 6 and 9 months. Homogenates of footpads containing more than 10^8 AFB were inoculated intravenously into four nine-banded armadillos. Noncultivable (in conventional media) AFB harvested from the footpads of nu/nu mice or sphagnum substrate were examined by dopaoxidase¹⁶ and pyridine decoloration¹² tests. The two homogenates examined in this way contained 3.7 and 3.5×10^7 AFB per ml, respectively, and were homogenized in 0.1% albumin and lyophilized without further purification. Lipid extracts were analysed by a chromatographic separation of the lipids followed by screening, using monoclonal antibodies specific for major *M. leprae* phenolic glycolipids.^{19,20}

Results

The water samples contained counts of AFB ranging from 10^2 to 10^5 per ml and the soil samples contained in most cases 10^5 per g (Table 2). A total of eight

Table 1. Description of the water and soil samples collected in Bombay on 9 and 10 February 1981

Sample No.	Locality & sample description	pH	Water/soil Temp. °C	Sample size: filtered water/ml soil/g
1	Dharavi, water reservoir on the school roof, leaking water	7·11	26	600 ml
2	Dharavi, reservoir in the school, tap water	7·11	28	600 ml
3	Dharavi public, stored water-drum, road side	6·94	26	600 ml
4	Danda Kahr, drinking water, stored in copper pot	7·27	26	600 ml
5	Danda Kahr, bazar Galli, stored drinking water	6·93	30	600 ml
6	Kopari, leprosy colony Mulund, well water	7·37	26	800 ml
7	Kopari, leprosy colony Mulund, tap water from the well	7·24	26	800 ml
8	Kopari, leprosy colony Mulund, well water, another tap	7·28	26	1000 ml
9	Santa Cruz, Best Quarters, Chunabhatti drinking tap water, stored	7·14	26	1000 ml
10	Santa Cruz, Best Quarters, Chunabhatti soil sample, wet soil on the road side	7·40*	28	40 g
11	Santa Cruz, Best Quarters, Chunabhatti drained waste water along the road	ND†	ND	50 ml
12	Santa Cruz, Best Quarters, Chunabhatti, wet soil sample near waste water ditch	7·65*	30	75 g
13	Bharat Nagar (North) Bandra, stored tap water-washing water	7·03	28	800 ml
14	Bharat Nagar (Bandra) wet soil, house washing place	6·92*	29	85 g
15	BDD Chawl (Worli) tap water, stored in house	7·38	18	800 ml
16	BDD Chawl (Worli) dry soil sample from the street place	7·12*	30	45 g
17	Worli, Sea Corner, surface washing water flowing into the sea	7·04	29	800 ml

Samples No. 1,2,3,4,5,9,13 and 15: Public water supply of Great Bombay

* pH measured in water homogenates by processing in the laboratory

† Not determined

Figure 1. Greater Bombay and area covered by the Bombay Leprosy Project with the location of samples collected (A–G). Arrow indicates the finding of environment-derived *M. leprae*. Further explanation in Table 2.

samples contained strains of cultivable mycobacteria (Table 2). Some of those strains also survived in footpads. In one sample from soil (E), examination of footpads for AFB revealed repeatedly acid-fast bacilli which failed to grow on conventional media. The mean count per footpad of non-cultivable AFB increased from 4.7×10^5 in 6 months to 8.5×10^6 in 24 months. In nu/nu mice, after a mean inoculation of 8.5×10^3 per footpad, an increase to a mean of 2.4×10^9 was observed after 9 months. The swelling obtained after 6 months is shown in Figure 2. The test for specific phenolic glycolipid I was positive (Figure 3). Tests for dopaoxidase and pyridine decoloration were positive. In biological tests, nerve involvement in nude mice and systemic leprosy in one of the infected armadillos were found.

Table 2. Samples of water and soil collected in six slum districts and one leprosy colony with count of acid-fast bacilli (AFB) per ml and per g, respectively, and the species of mycobacteria isolated.

Slum District	Water sample		Soil sample		Species of mycobacteria
	Sample No.	AFB(x.10 ³)/g	Sample No.	AFB(x.10 ³)/g	
Dharavi (A)	1,2,3	0.83–42			<i>M. lactae</i> <i>M. thermoresistible</i> <i>M. obuense</i> and <i>M. phlei</i> none
Danda Kahr (B)	4,5	6.7–27			<i>M. thermoresistible</i>
Kopari Leprosy Colony (C)	6,7,8	43–590			<i>M. gordonae</i>
Chunabhatti (D)	9,11	2.4–80	10,12	4.8–320	none
Bharat Nagar (E)	13	0.14	14	420	<i>M. gadium</i> (water sample) <i>M. intracellulare</i> (soil sample)
BDD Chawl Worli (F)	15	7.0	16	650	<i>M. gilvum</i> (both samples)
Worli Sea Corner (G)	17	0.99			<i>M. gilvum</i> <i>M. sphagni</i>

Figure 2. Swelling of the inoculated right footpad observed 6 months following infection (the left footpad served as a control).

Figure 3. Phenolic glycolipid I (Ph I) in AFB harvested from inoculated footpads 6 months after inoculation (column 5). Column 1 and 6 phenolic glycolipid I (100 mg); column 2 positive leprosy serum; column 3 and 4 negative leprosy sera; column 5 footpad sample (E).

Discussion

The samples collected in Bombay 1981 have shown a variety of cultivable mycobacteria. Only in one sample could mycobacteria be found which did not

grow on conventional media, and which multiplied in normal and nu/nu mice like *M. leprae*. They cause leprosy in armadillo and nude mice. In this sample, antigens believed to be specific for *M. leprae* were detected. Apparently, this is the first reported isolation of environmentally-derived *M. leprae* in a leprosy endemic region.

The epidemiological implication of this finding would need further clarification. On one hand, the *M. leprae* strain identified may have been deposited at the spot some time ago from a leprosy patient by coughing or sneezing. In this case, the epidemiological relevance may be limited. On the other hand, the sample may represent a reservoir independent of deposit from human sources, and then the epidemiological implications would seem highly important.

However, the likelihood that the spot from where the sample was taken, was particularly infected from leprosy patients, was rather low; no patients were living in the nearest houses. Taking into consideration the epidemiological indications already mentioned, the finding seems to support the concept of environmental sources of infection in leprosy. Clarification of the issue needs further cooperative microbiological and epidemiological studies based on more simple but still reliable methods for the identification of *M. leprae*.

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References

- ¹ Arnold HL, Fasal P. *Leprosy, Diagnosis and Management*. 2nd ed. Charles C Thomas, Springfield, Illinois 1973, 7–12.
- ² Beerwert W. Mykobakterien in Viehränken und Oberflächengewässern. *Dtsch Tierärztl Wchschr.* 1973; **80**: 398–401.
- ³ Enna CD, Jacobson RR, Trautman Jr. and Sturdivant M. Leprosy in the United States (1967–1976). *Public Health Rep*, 1978, **93**: 468–473.
- ⁴ Irgens LM. Leprosy in Norway. *Lepr Rev*, 1980; **51**: Suppl 1, 1–130.
- ⁵ Irgens LM, Kazda J, Müller K. and Eide GE. Conditions relevant to the occurrence of acid-fast bacilli in sphagnum vegetation. *Acta path microbiol scand.* Sect B 1981 **89**: 41–47.
- ⁶ Irgens LM. The discovery of *M. leprae*. A medical achievement in the light of involving scientific methods. *Am J Dermatol*, 1984; **6**: 337–343.
- ⁷ Kazda J. Mycobacterium sphagni sp. nov. *Int J System Bacteriol*, 1980; **30**: 77–81.
- ⁸ Kazda J. Occurrence of non-cultivable acid-fast bacilli in the environment and their relationship to *M. leprae*. *Lepr Rev*, 1981; **52**: Suppl 1, 85–92.
- ⁹ Kazda J. Growth of *M. leprae* and other pathogenic and saprophytic mycobacteria in peat substrate. Proc. 2nd Internat. Symposium Peat in Agriculture and Horticulture, Bet Dagan, Israel. 1983; 35–45.

- ¹⁰ Kazda J, Irgens LM, Müller K. Isolation of non-cultivable acid-fast bacilli in sphagnum and moss vegetation by foot pad technique in mice. *Int J Lepr*, 1980; **48**: 1–6.
- ¹¹ Kirchheimer WF. Leprosy in nature? (personal communication, 1980).
- ¹² McCormick TG, Sanches RM. Pyridine extractability of acid-fastness from *M. leprae*. *Int J Lepr* 1979; **47**: 495–496.
- ¹³ Meyers WM, Walsh GP, Brown HL, Rees RJW, Convit J. Naturally acquired leprosy-like disease in the nine-banded armadillo. *J Reticuloendothelial Soc* 1977; **22**: 363–367.
- ¹⁴ Müller K, Kazda J, Irgens LM. Zum Vorkommen von Mykobakterien in Mooren. *Telma*, 1980; **10**: 245–252.
- ¹⁵ Noordeen SK. Infectivity of leprosy; in *A window of leprosy*. Gandhi Memorial Leprosy Foundation, Silver Jubilee Commemorative Vol. B R Chatterjee (ed). Statesman Commercial Press, Calcutta, 1978.
- ¹⁶ Prabhakaran K, Harris EB, Kirchheimer WF. Hypopigmentation of skin lesion in leprosy and occurrence of ordiphenoloxidase in *M. leprae*. In *Pigment cell*. Vol. 3, S Karger, Basel, 1975.
- ¹⁷ Sand A. Geschieht die Ansteckung der Lepra durch unmittelbare Übertragung? Mitteilungen und Verhandlungen der 2. *Leprakonferenz*. G Fischer, Leipzig. 1910; 39–46.
- ¹⁸ Walsh GP, Meyers WM, Bindford CH, Gerone PJ, Wolf RH, Leininger JR. Leprosy—a zoonosis. *Lepr Rev*, 1981; **52**: Suppl 1, 77–83.
- ¹⁹ Young DB, Khanolkar SR, Barg LL, Buchanan TM. Generation and characterisation of monoclonal antibodies to the phenolic glycolipid of *M. leprae*. *Inf Immunol*, 1984; **43**: 183–188.
- ²⁰ Young DB, Fohn MJ, Buchanan TM. Use of polysulfone membrane support for immunochemical analysis of a glycolipid from *M. leprae*. *J Immunol Meth*, 1985; **79**: 205–211.