JOURNAL OF BACTERIOLOGY, Oct., 1966
Vol. 92, No. 4

Printed in U.S.A.

Use of 3,4-Dihydroxyphenylalanine Oxidation in the Identification of *Mycobacterium leprae*

KOCHUKUNJU PRABHAKARAN AND WALDEMAR F. KIRCHHEIMER
U.S. Public Health Service Hospital, Carville, Louisiana

Received for publication 23 June 1966

Identification of acid-fast bacteria failing to grow on artificial culture media under known conditions as *Mycobacterium leprae* now depends on the results of two time-consuming tests. At relatively low ambient temperatures, several thousand leprosy bacilli multiply within 6 to 9 months to maximal numbers of several million in the mouse footpad. Gross lesions or invasion of adjacent or remote tissues are absent (C. C. Shepard, J. Bacteriol. **90**:1271, 1965). The other test requires intradermal injection of leprosy patients simultaneously with the heat-killed mycobacteria and with standard lepromin. (Shepard and Guinto, Intern. J. Leprosy **31**:353, 1963). Leprosy bacilli are the only known mycobacteria failing to evoke dermal responses in Mitsuda-negative leprosy patients. Both tests distinguish between the noncultivated species, *M. leprae* and *M. lepraemurium*. Since culture of *M. leprae* is now widely attempted, methods for rapid screening are desirable.

Until recently, metabolic properties of the leprosy bacillus were unknown. Lately, Prabhakaran (Ph.D. Thesis, Univ. Bombay, 1964) found that *M. leprae* from lepromatous skin nodules oxidizes 3,4-dihydroxyphenylalanine (dopa) to colored products. The color development could be observed within 15 to 30 min of incubation of *M. leprae* with dopa. Some cultivable mycobacteria (*M. tuberculosis* strains $H_{37}Rv$ and $H_{37}Ra$, *M. bovis* strain B.C.G., *Mycobacterium* species 607, *M. phlei*, *M. smegmatis*, and two mycobacteria isolated from cases of leprosy (Kedrowsky's bacillus and the ICRC bacillus of the Indian Cancer Research Centre) failed to oxidize this substance. Since the oxidation of dopa gives rise to pigmented products, the reaction could be visually assessed. With the cultivable mycobacteria employed, no development of color was observed even with 15 mg of bacterial protein, and spectrophotometric measurements showed no increase in absorbance over the controls.

Because skin itself contains dopa oxidase, it became necessary to test *M. leprae* separated from tissue such as spleen which, as our data show, is devoid of the enzyme. Spleen was removed aseptically from a case of lepromatous leprosy after death. Cultures of the spleen remained negative. The tissue was homogenized in a VirTis 45 homogenizer. The homogenizing flask was surrounded with ice in the cooling cup. Leprosy bacilli were separated from the homogenate by differential centrifugation (Prabhakaran and Braganca, Nature **196**:589, 1962). Dopa and its structural analogues, epinephrine and norepinephrine, were incubated with *M. leprae*. The reaction products were measured spectrophotometrically after centrifugation of the mixtures. (See Table 1 for conditions of experiment.) The bacilli used were intact organisms and sedimented on centrifugation. Supernatant fluids of mixtures containing bacteria without substrate had little absorbance. Indole-5,6-quinone produced from oxidation of dopa absorbs maximally at wavelength 540 mμ. Adrenochrome formed from the other compounds gave a broad peak from 460 to 520 mμ. The results are presented in Table 1.

TABLE 1. *Oxidation of dopa and catecholamines by Mycobacterium leprae*[a]

Substrate	Concn of substrate	Time	Wavelength	Absorbance	
				With bacilli	Without bacilli
	mg	*min*	*mμ*		
L-Dopa	2	120	540	.187	.010
L-Epinephrine	0.4	45	480	.230	.025
DL-Norepinephrine	2	120	480	.180[b]	.030

[a] Protein concentration of bacilli, 2 mg (*p*H 6.8); temperature, 37 C; volume 3 ml. After incubation, the reaction mixture was centrifuged for 45 min at 15,000 × *g* and absorbance of the supernatant fluid was measured.

[b] The supernatant fluid was diluted twice before taking the reading. Undiluted, it would have been .360.

Dopa was oxidized by unheated but not by heated (100 C for 15 min) *M. leprae*.

Homogenates of spleens of humans who had died suddenly and mouse spleen in amounts of 50 mg (wet weight) did not oxidize dopa. The organs were homogenized in an iced Pyrex glass tissue grinder. Spleens from three different nonleprous individuals were tested. Homogenates without dopa and heated homogenates with dopa served as controls. After incubation for 2 hr at 37 C, the reaction mixtures were centrifuged and absorbance of the supernatant fluids read at 540 mμ. The experimental samples showed no increase in absorbance over the controls. *M. lepraemurium* separated from mouse spleen, *M. balnei*, and five isolates (at one time thought to be leprosy bacilli) obtained from American Type Culture Collection, in amounts of 2 to 4 mg of protein, did not oxidize dopa. These findings indicate a high degree of specificity for dopa oxidase in *M. leprae*. Several isolates alleged to be *M. leprae* are now being assayed in this laboratory.

This investigation was supported by Public Health Service grant AI 03636 from the National Institute of Allergy and Infectious Diseases.