Metabolic and Biologic Tests on Mycobacteria Once Labeled as Leprosy Bacilli[1, 2]

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Mycobacterium leprae separated from lepromatous skin nodules and from infected human spleen oxidizes 3,-4-dihydroxyphenylalanine (dopa) to indole-5,6-quinone, which can be determined spectrophotometrically ([4, 6]). Several mycobacterial species listed in Bergey's *Manual of Determinative Bacteriology*, 7th edition, 1957, like *Mycobacterium tuberculosis*, strains H37Rv and H37Ra ([4, 6]), *M. bovis*, strain BCG (Rosenthal) ([6]), *M. ulcerans* ([3]), *M. microti* ([3]), *M. lepraemurium* ([6]), *M. phlei* ([4]), *M. smegmatis* ([4]), *M. fortuitum*, and *M. marinum* ([3]) fail to do so. These findings indicate that among mycobacteria, dopa-oxidation is characteristic of the leprosy bacillus. Shepard ([7]) has shown that limited multiplication in the mouse foot pad at relatively low ambient temperatures, absence of gross lesions from the foot, and failure to invade adjacent or remote tissues, are characteristics of the leprosy bacillus. The mouse foot pad test also serves to distinguish leprosy bacilli from other mycobacteria.

It seemed of interest to subject mycobacterial species selected from the *Catalogue of Cultures*, 7th edition, 1964, American Type Culture Collection (ATCC), and believed at one time to be the leprosy bacillus, to the dopa-oxidase and the mouse foot pad tests. Also tested in the same way were some mycobacterial species listed in the *Catalogue*, some of which had been isolated originally from ticks experimentally infected with leprous material, and two others isolated from a leprous patient and from a guinea-pig, respectively.

MATERIALS AND METHODS

Mycobacterial species. The following mycobacterial "species," obtained from the ATCC in lyophilized condition, were used in the experiments: *Mycobacterium* sp. 4233, 4234, 4235, 4237, 4238, 4239, 4240, 4241, 4243, 4244 (these were designated *Bacillus leprae* by the depositor); *Mycobacterium* sp. 9031 and 9032, isolated by H. C. de Souza-Araujo from a leprous patient and from a guinea-pig, respectively; and *Mycobacterium* sp. 9033, 9034, and 9035, isolated from ticks experimentally infected with leprous material.

The lyophilized material was suspended in brain–heart infusion broth and incubated at 37°C in slanted tubes containing the same medium until a surface pellicle became visible. Part of the pellicle was transferred by means of a wire loop to the surface of 75 ml. of Proskauer-Beck medium contained in 250 ml. Erlenmeyer flasks. The cultures were incubated at 37°C until the growth had spread over the entire surface of the synthetic culture medium. In preparation for the dopa-oxidase and mouse foot pad tests the bacteria were separated from the growth medium by centrifugation and washing twice in normal saline. Acid-fast stains were made of films prepared from the sedimented bacteria.

Dopa-oxidase test. The intact mycobacteria in quantity representing at least 2 mgm. of bacterial protein were incubated in phosphate buffer, pH 6.8, with 2 mgm. of L-dopa at 37°C for 120 minutes. At pH 6.8 of the medium there is no auto-

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oxidation of dopa in two hours ([1]). After incubation, the reaction mixture was centrifuged for 45 minutes at 15,000 x g, and absorbance of the supernatant fluid was measured in a Beckman DU spectrophotometer, at a wavelength of 540 mμ, at which indole-5,6-quinone, produced in the oxidation of dopa by *M. leprae* absorbs maximally. Controls included the same reaction mixture, except that the bacteria had been omitted or had been heated for 15 minutes at 100°C. As a check on the method, identical tests with dopa-oxidase-positive leprosy bacilli, obtained from lepromatous spleens, were performed at times. It was impractical to use leprosy bacilli from skin lepromas, as sufficient amounts of skin lepromas were not available. In addition, leprosy bacilli from spleen are preferable, since spleen itself does not contain dopa oxidase. Since the reaction gives rise to pigmented products, the activity could also be assessed visually.

Mouse foot pad tests. Bacterial suspensions were prepared by grinding some of the washed bacterial mass in a mortar with slow additions of Hanks' balanced salt solution (BSS). Bacterial clumps were removed by centrifugation. The number of bacteria in the supernatant was determined by counting according to a modification ([2]) of the technic of Hanks, Chatterjee, and Lechat ([1]). The bacterial suspension was finally diluted with BSS so that the volume of 0.03 ml. injected into the mouse foot pad contained the desired number of bacteria, which ranged from 3 x 10^3 to 48 x 10^3. For each respective mycobacterium one dose level only within this range was inoculated into 20 mice of either the NIH utility or the Swiss-Webster strain. Ten of these mice per cage were housed in air-conditioned quarters with ambient temperature around 20°C, and the remaining 10 mice per cage at room temperature, ranging from 30°C during the warmer months to 20°C during the cooler ones.

At the same time groups of mice were inoculated with comparable amounts of *M. leprae* from patients with lepromatous leprosy.

RESULTS

Dopa-oxidation. None of the 15 mycobacterial species from the ATCC oxidized dopa even when bacterial masses were used far in excess of those of *M. leprae* that regularly give a positive test. A permeability barrier could be excluded since bacilli crushed with sand in an agate mortar also gave the same results.

Mouse foot pad tests. Several mice of each group were sacrificed three months after inoculation. Their inoculated feet were removed and fixed in formaldehyde for at least one week. They were then decalcified at room temperature in a solution of 5 per cent formic acid in 70 per cent ethanol. After embedding in paraffin, sections 6 to 8μ thick were cut, and stained with acid-fast and hematoxylin-eosin stains. Acid-fast stains were also prepared from sections of the livers, spleens, and lungs of these mice. In no instance were acid-fast organisms, other bacteria, or histologic changes seen in any of these preparations. Additional animals from each group were sacrificed six months after inoculation. Their foot pads were removed with a scalpel and minced with scissors in a mortar. The tissue then was ground with a pestle, with slow addition of BSS. Counts of acid-fast bacteria were made from these suspensions, as stated above. Among the ATCC mycobacteria assayed, indication of multiplication was obtained only in the case of species 4238, when the mice were housed in the air-conditioned quarters. These animals had been inoculated with 9.4 ± 0.6 x 10^3 bacteria. Six months later 28.0 x 10^3 acid-fast bacteria per foot pad were recovered. There was, however, no sign of multiplication in the foot pads of mice inoculated with species 4238 and kept at a higher ambient temperature. These animals also had received 9.4 ± 0.6 x 10^3 acid-fast bacteria and the count after six months was $<$11.0 x 10^3 bacteria per foot pad. On the other hand, mice that had been inoculated at the same time with from 8.5 ± 0.4 x 10^3 to 33 x 10^3 leprosy bacilli from Carville patients had, 172 to 281 days later, 28 to 67 times as many

TABLE 1. *Multiplication of* M. leprae *from lepromatous skin lesions of human beings in foot pads of NIH strain mice.*

No. of bacteria inoculated	No. of mice sacrificed	Average No. of acid-fast bacteria harvested per foot pad	Days after inoculation	Bacterial increase
20×10^3	8	550×10^3	226	28 fold
20×10^3	3	470×10^3	226	23 fold
8.5×10^3	4	250×10^3	172	30 fold
9×10^3	4	600×10^3	281	67 fold
12×10^3	4	700×10^3	280	58 fold
33×10^3	6	$2,200 \times 10^3$	180	66 fold

bacteria in their foot pads (Table 1). The multiplication rate of these leprosy bacilli was probably much greater than these data indicate, because less than 10 per cent of the inoculated bacilli displayed the tinctorial and morphologic characteristics of viability. For example, in the case where 8.5 x 10^3 leprosy bacilli had multiplied 30 fold within 172 days the calculated generation time is 35 days. However, since the inoculum contained only 7.6 x 10^2 viable bacilli a generation time of 20.7 days might be more nearly correct.

DISCUSSION

The results of the dopa-oxidase and mouse foot pad tests show that none of the 15 mycobacterial strains behaved like *M. leprae*. Multiplication in the mouse foot pad occurred only with *Mycobacterium* sp. 4238, but was much slower than is typical for *M. leprae* under the same conditions. This mycobacterium achieved only approximately 1.5 generations during the time when leprosy bacilli produce approximately 9 generations. It has been questioned recently if the dopa-oxidase activity of leprosy bacilli might not result from enzymes present in the cutaneous tissue of the host, and if the leprosy bacillus might carry these enzymes to its secondary location in the spleen, which itself is devoid of dopa-oxidase ([6]). If this were true, search for dopa-oxidase activity would not be a valid test for mycobacteria grown in artificial culture media. This possibility, however, has been excluded, since it was shown that the respective enzymes of the leprosy bacillus and of mammalian tissue are different and, therefore, distinguishable ([5]).

The results of these studies thus demonstrate that the mycobacterial cultures, some of which were originally believed to be *Bacillus leprae*, do not possess the metabolic and biologic properties of the leprosy bacillus.

SUMMARY

Fifteen mycobacterial strains from the American Type Culture Collection, 10 of them originally designated as *Bacillus leprae*, were subjected to the dopa-oxidase and mouse foot pad tests. None of these mycobacteria was able to multiply in the mouse foot pad in a manner typical for *Mycobacterium leprae*, and none of them oxidized dopa, as does the leprosy bacillus.

RESUMEN

Quince cepas de micobacterias de la American Type Culture Collection, 10 de los cuales originalmente rotuladas como *Bacillus leprae*, fueron sometidos a las pruebas de dopa-oxidasa y colchón plantar de raton. Ninguno de estas micobacterias pudo multiplicarse en el colchón plantar de raton de la manera característica propia del *Mycobacterium leprae*, y ninguno oxidó dopa, como lo hace el bacilo de la lepra.

RÉSUMÉ

Quinze souches mycobactériennes appartenant à l'American Type Culture Collection, parmi lesquelles 10 avaient d'abord été étiquettées comme *Bacillus leprae*, ont été soumises à l'épreuve de la dopa-oxidase, et à celle de la sole plantaire de la souris. Aucune de ces mycobactéries ne s'est révélée capable de multiplication dans la sole plantaire de la souris d'une manière qui puisee être considérée comme typique de *Mycobacterium leprae*, et aucune d'entre elles n'a oxydé la dopa, comme le fait le bacille de la lèpre.

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