Identification of the Noncultivable Pathogenic Mycobacteria *M. leprae* and *M. lepraemurium*[1,2]

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In the experimental attempts at transmission of human lepromatous leprosy to laboratory animals it is of great importance to distinguish the noncultivable *Mycobacterium leprae* from the equally uncultivable *M. lepraemurium*, as well as from the strains that may be viable in the animal host with or without adaptive mutation in the new environment. Two methods are available at present for such studies, viz.:

1. Administration to a lepromatous patient of an antigen prepared from the mycobacterial strain in a manner similar to the Mitsuda-Hayashi method. As is well known, the antigen prepared from the bacilli found in lepromatous human lesions produces no reaction in the lepromatous patient. However, it is a long and indirect procedure, which will often depend on the presence, in the vicinity, of lepromatous cases as a source of bacilli.

2. The Prabhakaran method (2), which demonstrates that *M. leprae* oxidizes 3,-4-dihydroxyphenylalanine after 15 to 30 minutes' incubation. Because of the normal presence of dopa oxidase in the skin, splenic tissue is used, and by means of homogenization, differential centrifugation, and spectro-photometry, the usefulness of the method for *M. leprae* and its differentiation from *M. lepraemurium* are determined. This method, however, has been criticized with respect to its specificity (3). Furthermore, it involves difficulties in performance, and can be carried out only in a fully equipped laboratory.

The main purpose of the research here reported has been to develop a cytochemical method that will allow differentiation of the noncultivable mycobacteria among themselves, and specifically of *M. leprae* and *M. lepraemurium*.

MATERIALS AND METHODS

In our primary experiments with Baker's histochemical test, which is highly specific for phospholipids (1), we used 11 nodules from the skin of eight treated and three untreated lepromatous patients. The same number of peritoneal and dermic lepromas were secured from rats and mice inoculated with a strain of *M. lepraemurium*, which Dr. Y. T. Chang of the National Institutes of Health, Bethesda, Maryland, had kindly furnished us. In our investigation of the applicability of the test to mycobacteria we used two sets of preparations for each strain or species. One set was submitted directly to the method of the test, while the other was first submitted to the Baker process for the removal of phospholipids and thereafter to the test following the same steps as with the unextracted set. The materials studied were (1) sections from human lepromatous tissue and from leprous rat tissue, both cut to a thinness of 8-10 millimicrons, and (2) smears from homogenized preparations of the same tissues. Later on we also used simple

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lymph smears from human as well as animal sources of bacilli.

The experience gained in the primary experiments soon convinced us that certain modifications of procedure and timing were necessary in order to obtain the best visualization of the bacilli in the smears. The Baker test for phospholipids, as modified by us for use with smears, is carried out as follows:

1. Dry the smears on cover slides at room temperature for 4-5 hours or in an oven for 1 hour at 37°C.
2. Fix the smears in Baker's formol-calcium for 5 hours.
3. Leave the cover slides overnight or for 16 hours in a 5% calcium dichromate solution.
4. Rinse with distilled water. (Steps 3 and 4 are optional).
5. Place the cover slides in a mordant solution of calcium dichromate for 1 hour at 60°C.
6. Rinse with distilled water.
7. Stain in acid hematein at 60°C for 1 hour.
8. Rinse in distilled water.
9. Differentiate in boric ferricyanide for 1.5-2 hours.
10. Rinse in distilled water.
11. Blot dry.
12. Mount the cover slides with Permount on the unstained side of the slide and observe directly with oil immersion.

The Baker technic for the removal of phospholipids, as adapted by us, is the following:

1. Fix in Bouin's fluid for 5 to 16 hours.
2. Place the cover slides in 70% alcohol for 5 minutes.
3. Place them in 50% ethyl alcohol for 5 minutes.
4. Rinse in tap water for 1-2 minutes.
5. Dehydrate in fresh pyridine for 2 hours.
6. Rinse in tap water for 5 minutes.
7. Continue with step No. 3 from Baker's method for smears as already outlined.

As a complementary procedure we used the PAS reaction (Tomassi-Schiff) on frozen sections, using the same formol-calcium as a fixative. A similar study was made with the use of cultivable mycobacteria, such as *M. fortuitum*, the bacillus Calmette-Guerin (BCG), *M. chromogenum hominis*, *M. balnei*, *M. tuberculosis* H37Ra and *M. butyricum*, using the Baker as well as the PAS technic.

RESULTS

Human lepromas and smears. Under low magnification the sections of human lepromatous tissue showed a granuloma of intense dark-blue color, which would indicate the presence of phospholipids or mucins or both, but as the blue color was not found in the extracted sections, it could definitely be attributed to phospholipids only. On higher magnification the blue masses were seen to be located in the cytoplasm of mononuclear cells or histiocytes. The immersion showed that the blue masses were made up of bacilli, some intact and some granular, the blue color indicating that they must have contained very large amounts of phospholipids (Fig. 1). The unextracted smears prepared from homogenized lepromatous tissue, as well as the simple lymph smears, revealed isolated, intact or granular bacilli or globi stained a dark blue, but the blue coloration was absent in the extracted smears. Here again we had proof that the stain was retained exclusively by phospholipids in *M. leprae* (Fig. 2).

Animal lepromas and smears. Our study of sections made from murine peritoneal lepromas revealed something remarkably different. The modified Baker test applied to those sections produced indistinct dark-blue granular or bacillary forms in the cytoplasm. In the extracted sections the blue color not only persisted, but was actually intensified and better defined (Fig. 3). Smears from homogenized murine lepromas and lymph smears from the same source showed blue-stained bacilli, in both the extracted and the unextracted slides. The PAS test was positive in the smears of the cultivable as well as the noncultivable mycobacterial species to which it was applied, such as *M. leprae*, *M. lepraemurium*, *M. balnei*, *M. fortuitum*, BCG, *M. tuberculosis* H37Ra, and *M. chromogenum hominis*.

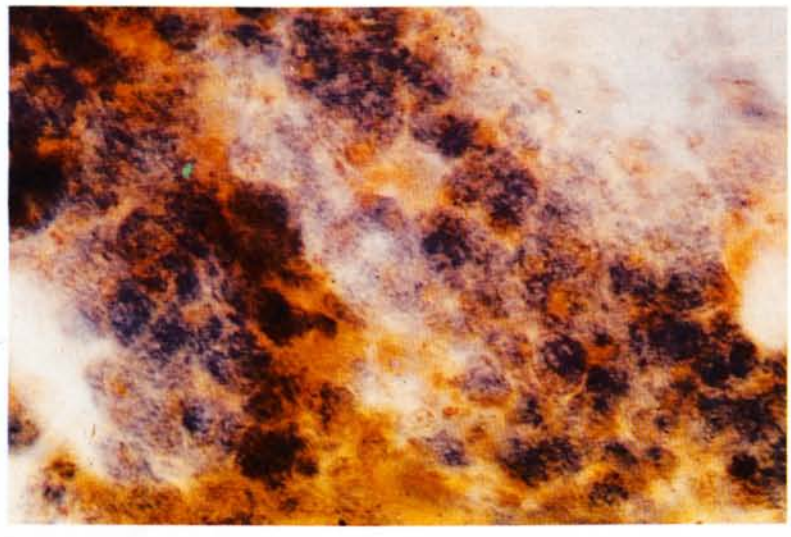

FIG. 1. Section of leproma from case of lepromatous leprosy, showing the bacilli stained a deep dark blue (Baker's technic).

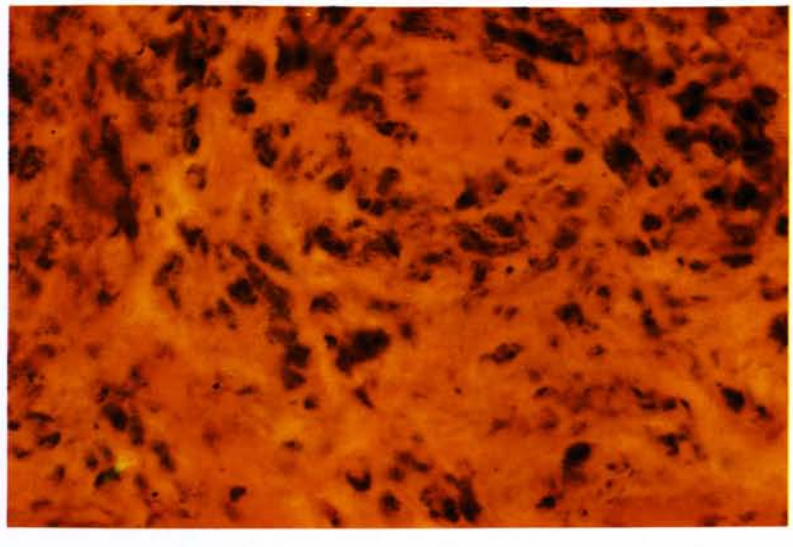

FIG. 2. Section of murine peritoneal leprous lesion after pyridine extraction, showing the bacilli stained a deep blue.

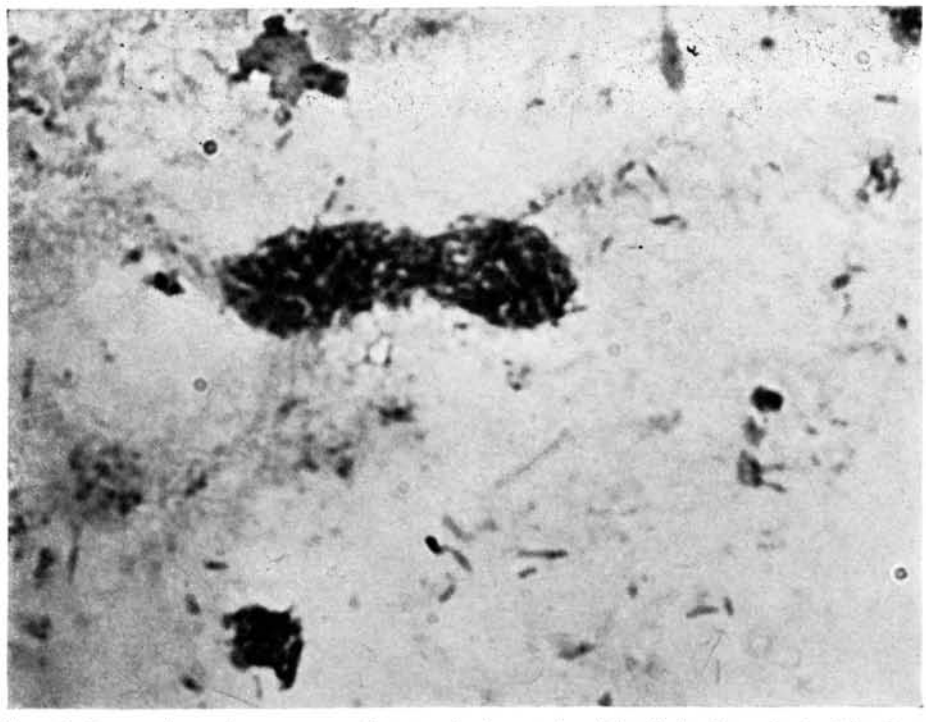

FIG. 3. Smear from lepromatous leprous lesion stained by Baker's technic. The bacilli and globi are stained dark blue.

DISCUSSION

The use of Baker's technic for phospholipids, with the modifications employed by us in smears, fills a need in the experimental field for differentiating *M. leprae* from *M. lepraemurium*. The studies carried out demonstrated that *M. leprae* is the only one of the noncultivable mycobacteria used, including the noncultivable types, that produces a clear and definite coloring in the Baker method for phospholipids.

The results obtained from tissue sections of murine leprosy demonstrate that the content of polysaccharides in these bacilli, and not the phospholipids, is responsible for the coloring obtained, since the blue color not only remained but was intensified upon extraction. We believe that the coloring of the phospholipids is an exclusive property of the *M. leprae* of human origin, which could be applied in the study of mycobacteria observed in experimental inoculation of human leprosy in laboratory animals or in a possibly successful culture. Another possible use of this method could be for the identification of *M. leprae* among the mycobacteria found in healthy persons originating from endemic areas of leprosy. It could also be applied to the study of the morphologic index of the *M. leprae*.

SUMMARY

A new and easily performed histochemical method for the identification and differentiation of two noncultivable mycobacteria, *M. leprae* and *M. lepraemurium*, is presented.

Baker's method for phospholipids is used primarily and for their concomitant verifying extraction in tissue sections, as well as smears. In the latter our modified version of the Baker method was used.

The PAS technic, applied to the cultivable and noncultivable mycobacteria, gave positive results in all of our studies.

The importance of use of the method for its epidemiologic value in healthy carriers is stressed.

RESUMEN

Se presenta un nuevo método histoquímico fácil de ejecutar para la identificación y diferenciación de dos micobacterias, no cultivables, *M. leprae* y *M. lepraemurium*.

Se usó primariamente el método de Baker para fosfolipidos, y concomitantemente y con propósitos de verificación se extrajeron trozos de tejidos, asimismo se prepararon frotis. En últimos, se uso nuestra versión modificada del método de Baker.

La técnica de PAS, aplicada a las micobacterias cultivables y no cultivables, dió resultados positivos en todos nuestros estudios.

Se pone énfasis en la importancia del uso del método por su valor epidemiológico en portadores sanos.

RÉSUMÉ

On présente ici une nouvelle méthode histochimique d'un emploi facile pour l'identification et la différenciation de deux mycobactéries non cultivables, *M. leprae* et *M. lepraemurium*.

A la base, on utilise la méthode de Baker pour les phospholipides et pour leur extraction concomitante et vérifiée dans les coupes de tissus, ainsi que dans les frottis. Pour ce dernier procédé, les auteurs ont utilisé leur version modifiée de la méthode de Baker.

La technique du PAS, appliquée tant aux mycobactéries cultivables que non cultivables, a fourni des résultats positifs dans toutes les études menées par les auteurs.

On insiste sur l'importance que représente l'utilisation de cette méthode, vu sa valeur épidémiologique chez des porteurs sains.

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