

THE BACTERIOLOGIC DIAGNOSIS OF EARLY LEPROSY  
BY THE TRYPSIN DIGESTION METHOD<sup>1</sup>

JOON LEW, M.D.  
AND MIN CHUNG, M.D.

*Department of Microbiology  
Yon-Sei University College of Medicine  
Seoul, Korea*

In many cases in the very early stages of leprosy the clinical symptoms and the histopathologic findings are not specific enough to make clear the diagnosis, and no specific serologic or immunologic test is available. As yet, detection of *Mycobacterium leprae* from suspicious leprosy lesions is the only key to definitive early diagnosis.

Many attempts have been made to concentrate the bacilli from leprosy tissues. In 1897, Alvarez (<sup>1</sup>) described a new method of bacteriologic diagnosis of obscure cases of leprosy, in which a bit of skin was removed and ground up in a small amount of saline. If bacilli could not be found in direct smears of the triturate, it could be centrifuged or (lacking a centrifuge) be allowed to sediment. As a last resort when bacilli could not be found, he suggested, "digestive ferments" might be added. This is believed to be the first mention of this possibility in the literature.

In 1952, Khanolkar and Rajalashmi (<sup>2</sup>) described a method of concentrating bacilli from specimens of skin lesions which were negative by ordinary methods of examination. This procedure involved separation of the bacilli from the suspension of the tissue specimen with petroleum ether. Dharmendra and Mukherjee (<sup>4</sup>) employed chloroform for the same purpose.

There are two different objectives in concentrating bacilli from the lesion tissue, one to find them when they are scarce, as in the work cited and that to be reported here, the other to separate them from the tissue elements so that antigenic preparations can be made of them by weight, as in the work of Fernandez and Olmos Castro (<sup>5</sup>) and Dharmendra (<sup>2, 3</sup>). The former authors salted the leproma suspension and centrifuged out the tissue particles, and then reduced the specific gravity with alcohol and threw down the bacilli. Dharmendra extracted the bacilli directly from the leproma with chloroform.

Our own work in this field began about 1943 with the same objective, i.e., to collect bacilli relatively free of tissue debris. This was reported at the 18th Japanese Leprosy Conference, in 1943 (<sup>6</sup>). Work along the

<sup>1</sup>An abstract of this paper was sent to the VII International Congress of Leprology, held in Tokyo November 12-19, 1958, but another paper was chosen for the program.

same line was resumed in California in 1953<sup>(9)</sup>. It was recognized that the main obstacle to successful collection of the bacilli was the occurrence of globi and bundles of tightly-packed leprosy organisms which in size and specific gravity were similar to some of the tissue particles, such as nuclei. Thus the globi could not be separated from the ground tissue by centrifuging alone.

To overcome this obstacle the tissue specimens were coarsely ground or cut with the freezing microtome into slices about ten microns thick, and placed in large tubes suspended in saline. The tubes were rapidly frozen by placing them in a mixture of dry ice and alcohol, and then thawed in a 37°C water bath. This freezing-thawing process was repeated many times in order to separate the bacilli in the globi as well as to break up the tissue cells. During the process film specimens were prepared to determine the effectiveness and progress of the procedure.

When tissue-cell breakdown was thought to be complete, the tubes were centrifuged at 500 rpm for 5 minutes to eliminate the large tissue particles. The suspension was drawn off and an equal volume of toluol was added to it. Various other fat solvents, such as ether, chloroform and petroleum fractions were tested, but toluol seemed to have the least effect on the morphology and staining behavior of the bacilli. The toluol mixture was shaken for 20 minutes in a shaking machine, and the milky mixture was then centrifuged at 1,500 rpm for 15 minutes.

Three layers formed in the tubes. The upper layer was clear toluol; the lower layer was turbid water containing small tissue particles; the middle layer was a thin milky ring of acid-fast bacilli. The bacterial layer was easily removed with a pipette.

Subsequently, attention was turned to the use of proteolytic enzymes to digest the tissue elements of the leproma suspension, and that led in due course to the present study. Lew<sup>(10)</sup>, and Lew and Carpenter<sup>(11)</sup>, after having tested many such enzymes, reported that trypsin had proved the most effective agent for the purpose, leaving the bacilli intact. Stefansky bacilli obtained from rat lepromas by this method proved to be still infectious for rats, whereas Nakamura<sup>(12)</sup> has reported that bacilli concentrated by the chloroform method were no longer infective.

It was suggested that this method might be useful in the diagnosis of leprosy, by examination of smears of the deposit after centrifuging. Under the circumstances, however, it was not possible to supply actual data based on the examination of early leprosy cases, so the practical application of this technique could not be proved. Now, from Korea, where leprosy is fairly prevalent, the following data are available for report.

#### MATERIAL AND METHOD

A total of 175 cases, divided into three groups, have been examined by this method.

*Cases examined.*—GROUP I: Forty-six slightly suspicious cases, having skin patches with slight alteration of pigmentation or of surface texture. Most of these patients were found during a routine survey and themselves had no suspicion that they might have leprosy. We were not able to make a diagnosis of leprosy by ordinary methods.

GROUP II: Twenty-nine cases which were clinically diagnosed as early leprosy, showing early lesions with anesthesia and altered skin pigmentation, with or without nerve thickening.

GROUP III: One hundred cases which had been confirmed as closed cases by Wade's scraped incision method (13). They had received prolonged treatment at the So-Rok-Do National Leprosarium, some of them for many years.

A suspected lesion was first examined by the scraped incision method and then a small biopsy specimen was removed. This was divided into two parts, one to be sectioned for histopathology and acid-fast staining, the other for digestion.

*Digestion method.*—The tissue specimen, about 0.5 gm. in weight, freshly obtained or formalinized, was autoclaved at 15 pounds for 15 minutes. It was then ground up in a mortar in 7-8 cc. of Sørensen's buffered solution (pH7.8) containing 0.5 per cent trypsin (Difco 1:250). The preparation was then incubated with frequent agitation at 39°C for 3 hours. Subsequently it was centrifuged at 3,000-4,000 rpm for 30 minutes, and the supernatant fluid decanted. The deposit was then examined for acid-fast organisms. If the tissue had been formalinized for transportation or storage, it was washed for 6 hours in running water before autoclaving.

#### RESULTS

The over-all results of these examinations are shown in Table 1, in comparison with the findings by the scraped incision method and histopathology.

TABLE 1.—Comparison of findings by the trypsin digestion method compared and the scraped incision method.

Case group	No. of cases	Trypsin incision method		Scraped digestion method	
		Positive	Negative	Positive	Negative
I	46	12 (26.1%)	34 (73.9%)	0 ( 0.0%)	46 (100%)
II	29	28 (96.6%)	1 ( 3.4%)	13 (44.9%)	16 (55.1%)
III	100	21 (21.0%)	79 (79.0%)	0 ( 0.0%)	100 (100%)

TABLE 2.—Details of findings in the 29 early cases classed in Group II, and 5 pure neural cases.

Type of lesion	No. of cases	Trypsin digestion method		Scraped incision method	
		Positive	Negative	Positive	Negative
Lepromatous patches	7	7 (100%)	0 ( 0.0%)	6 (85.7%)	1 (14.3%)
Tuberculoid or indeterminate patches	22	21 (95.5%)	1 ( 4.5%)	7 (31.8%)	15 (68.2%)
Pure neural cases <sup>a</sup>	5	0 ( 0.0%)	5 (100%)	0 ( 0.0%)	5 (100%)

<sup>a</sup> Cases with anesthesia but no other skin involvement.

Group I: In 12 of the 46 slightly suspicious cases, or 26.1 per cent, acid-fast organisms were found by the trypsin digestion method, but in none of them were bacilli found by the other methods of examination.

Group II: In all but 1 of these clinically diagnosed cases—28 of the 29, or 96.6 per cent—acid-fast bacteria were found by the digestion method. The scraped incision method gave positive findings in only 13 cases, or 44.9 per cent.

Group III: From these 100 old cases, bacteriologically negative by the usual method of examination, 21 (21%) of the specimens were positive for acid-fast bacteria.

The findings in Group II, a miscellaneous lot of early cases, are given in detail in Table 2. It will be seen that the digestion method revealed acid-fast bacilli in all of the 7 cases with patches diagnosed as lepromatous, whereas 1 was negative by the ordinary method. The most striking findings are in the 22 cases of the early tuberculoid-indeterminate group. With the digestion method all but 1 were positive, or 95.5 per cent, against only 31.8 per cent by the scraped incision method.

Besides the cases discussed, we examined a few pure neural cases without skin involvement. Although there were only 5 such cases, it is of interest that neither method revealed bacilli in any of them in the skin specimens examined.

#### DISCUSSION

Identification of leprosy in the advanced stages is not difficult, but early diagnosis is often most difficult. However, it is very important to make the diagnosis early, while the case is still noninfectious or only mildly infectious and can respond well to present-day chemotherapy.

Wade's scraped incision method for demonstrating the Hansen bacillus is simple and convenient, but technically it is limited to bacilli which are in the scraped area alone. The examination for acid-fast organisms in sections is only for those in a very thin layer of the lesion, and the technique is rather tedious and time-consuming. At this laboratory stage of leprosy work, it is desirable to have a technique in which the bacilli can be separated from the tissue elements and concentrated in a film specimen, as are tuberculosis bacilli in sputum.

Attempts to accomplish that end have been made by many workers since the time of Alvarez, and these attempts can be grouped in three categories: (1) Simple grinding of the leprosy tissue and centrifugation [e.g., Hanks (6)]. (2) Separation of the bacilli with an organic solvent from triturated leprosy tissue and centrifugation at different speeds (4, 7, 8). (3) Digestion of the tissue part of the leprosy suspension, leaving the bacilli intact, and centrifugation at different speeds (10, 11).

With the first of these three methods it is impossible to get rid of tissue debris completely, and the chances are that many organisms will be lost during the centrifugation. In the second method, the organic solvents decrease the acid-fastness and alter the chemical structure of the organisms, and at the same time there is always a great amount of acid-fast particulate matter besides the bacilli and it is very difficult to differentiate them. In the third method, the proteolytic enzyme digests almost all of the cell components and the bacilli are left intact. As said, the viability and acid-fastness of *M. leprae murium* were proved intact after exposing them in the trypsin solution for 5 hours at 39°C. (<sup>10, 11</sup>), whereas they are rendered noninfective by the chloroform extraction method (<sup>12</sup>). In this technique the protein part of tissue is well digested, and the fatty material is removed by centrifuging when the organisms are concentrated. In one of our experiments, 1 acid-fast organism per 40-50 microscope field became concentrated to 5-10 organisms per field. This is naturally the result of the concentration of the organisms from all parts of a whole biopsy specimen.

#### CONCLUSIONS

1. The trypsin digestion method described has revealed acid-fast bacilli in 26 per cent of suspect cases which otherwise would have been impossible to diagnose with certainty.
2. This method demonstrated acid-fast bacilli in almost 100 per cent of clinically diagnosed, untreated leprous lesions.
3. The technique and instruments required for this method are very simple. Wherever an ordinary centrifuge, a waterbath, and trypsin are available, the method can be employed.
4. The method can be used to demonstrate bacilli in formalin-preserved tissue specimens. Consequently, specimens of suspicious lesions can be sent to a central laboratory for examination.

#### CONCLUSIONES

1. La técnica aquí descrita para la digestión de la tripsina ha revelado bacilos ácidosresistentes en 26 por ciento de los casos sospechosos que de otro modo hubiera sido imposible diagnosticar con certeza.
2. Este método descubrió bacilos ácidosresistentes en casi 100 por ciento de las lesiones leprosas no tratadas, diagnosticadas clínicamente.
3. La técnica y los instrumentos necesarios para este método son muy sencillos. Dondequiera haya una centrífuga ordinaria, un baño-maría y tripsina, puede emplearse el método.
4. Puede usarse el método para descubrir bacilos en ejemplares histológicos conservados con formalina. Por consiguiente, pueden

enviarse muestras de lesiones sospechosas a un laboratorio central para examen.

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