LEPROMIN VS PURIFIED BACILLUS SUSPENSION
I. PREPARATION OF A PURIFIED BACILLUS SUSPENSION (WITH A NOTE ON NILE-BLUE STAINING OF SMEARS)[1]

H. W. Wade, M.D.

Pathologist Emeritus, Leonard Wood Memorial
Culion Sanitarium, Philippines

During the many years the lepromin test has been in use many attempts have been made, without notable success, to obtain an antigenic preparation without the tissue elements present in the classical Hayashi-Mitsuda "vaccine." The need for such a "purified" antigen has been felt less by the practical workers than by the theorists, who—quite understandably—dislike the "crude" lepromin. Undoubtedly, however, it would be preferable to use a purified preparation with which there would be no uncertainty about what element is active, provided one can be developed which is equally effective, and practicable to manufacture. This matter is still being discussed in certain quarters. Although the work which resulted in production of the "purified bacillus suspension" here described was done nearly five years ago and has been cited ([22]), it is now reported in detail. In a paper to follow will be the results of the clinical tests made with the purified bacillus suspension.

In considering the problem there are at least three possible approaches, namely extracting the bacilli from the original tissue with an oil, or doing that with a substance such as chloroform or some other fat-solvent reagent, or removing the tissue elements by a digestive enzyme. Prime requirements are reasonable simplicity of the method of preparation, so that it can be used generally; and, even more important, economy of the leproma tissue, which in these days of sulfone treatment is often in short supply whereas the use of lepromin is increasing.

Considerations of economy eliminate such wasteful procedures as the "fractional centrifugalization" method once reported by Dharmendra ([4]), or the more elaborate process used by Hanks ([8]) to obtain suspensions of the murine bacillus for metabolism studies. The same is to be said of the method of Fernandez and Olmos Castro ([7]), in which the specific gravity of the suspension was altered by first adding salt to float the bacilli and then alcohol to permit centrifuging them out.

Back in 1924, Mudd and Mudd ([16]) began a fundamental study of the stability of particles and bacteria in watery-organic interfaces. They observed that while ordinary bacteria (the surface of which contain many active or polar groups, and so are readily wet with water, the polar liquid) remain in the watery phase, acid-fast bacteria (which are coated predominantly with nonpolar substances) have very low or no stability in the interface and consequently pass easily or even spontaneously out of the polar watery phase into the organic phase. For the latter phase, various oils were used (cedar, peanut,

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mineral, etc.). In 1931, Reed and Rice [19] extended the study with acidfasts, using oils (including olive and coco) and also various fat solvents (including chloroform).

The first—and, so far as I am aware, the only—application of this principle with an oil to leprosy material was by Henderson [10], when he used olive oil to extract the bacilli from an aqueous suspension of spleen tissue, afterward washing the bacilli with acetone, which remained acidfast.

On the same principle, reagents such as ligroin, chloroform, xylol, etc., have long, if not commonly, been used for the concentration of the tubercle bacillus in materials such as sputum digests or cerebrospinal fluids. According to Smith [20], this was done as long ago as 1909 by Lange and Nitsche and by Bernhardt with ligroin, and in 1910 by Loeffler with chloroform. Other workers used other substances (gasoline, xylol, etc.), but Andrus and MacMahon [1] and Hanks and Feldman [9] found chloroform preferable. It goes to the bottom with all the bacilli, clumps of which are dispersed, and the sediment adheres well to the slide.

Dharmendra [5, 6] adopted chloroform for obtaining bacilli from the leproma tissue and, after "defatting" them (i.e., extracting the lipids, and acidfastness) with ether, made his "defatted" bacillary antigen. That method has been used rather widely, and seems still to be in use in some places. Lew [13] has told of finding toluol to have less harmful effects on the morphology and staining capacity of the bacilli, but that substance seems to have been given little attention. Nakamura [17] has used it, and several other substances (chloroform, amyl alcohol, benzene and benzine), but found the bacilli too much affected to be useful for chemical studies.

At the time the work to be reported here was under way Lowe [14], unable for lack of facilities to employ the entire Dharmendra process at Uzuakoli in Nigeria, evaporated off the chloroform after removing the bacilli from the leproma, suspended the gummy residue in phenol-saline by grinding in a mortar, and, after the fats had floated, drew off the suspension and "standardized" it. Later, however, Davey [3]—who took over at Uzuakoli after Lowe went to London—became dissatisfied with that product because of weak reactions and frequent negative findings in tuberculoid cases, and returned to the use of regular lepromin.

As for the use of enzymes, Dharmendra [4] and Chaussinand *et al.* [2] have mentioned using papain in such work, but they did not pursue the subject. Hanks is said (personal communication cited by Nakamura [18]) to have found pancreatin and bile to be useful, but he does not recommend it. After earlier work by Lew in Korea, Lew and Carpenter [12] used trypsin to separate bacilli in a process that was wasteful because larger particles (bacillus-containing tissue fragments and bacillary clumps) were discharged after centrifuging. More recently Lew and Chung [13] have used trypsin to demonstrate bacilli from tissue speciments in diagnostic work.

Recently, trypsin has been used by certain Japanese workers—Nakamura [18], Ito and Sonoda [11], Yanagisawa and Asami [23], and Mori, Kosaka and Ito [15]—for obtaining tissue-free suspensions of the murine bacillus in a viable state, and therefore uninjured. However, the process as shown in a flow chart [23] is elaborate, and since it involves high-speed centrifuging it would be quite impracticable in most places for preparing an antigen for lepromin-type testing; and it is not certain that it would be economical of leproma material.

EXPERIMENTAL WORK

In my own work a considerable amount of preliminary experimentation was done with numerous reagents, most of which would be pointless to recount.

First, chloroform was used for harvesting the bacilli, and attempts were made to remove the chloroform—and the substances (fats and

proteins) dissolved in or accompanying it—by Boerner (Seitz) filtration in the centrifuge[2], and by vacuum filtration with a fine-grade fritted-glass filter. These methods, however, proved impractical.

The expedient was then adopted of mixing aliquots of the chloroform-bacillus suspension with various substances of low specific gravity to permit depositing the bacilli quantitatively by centrifuging. Acetone proved to the best substance for this purpose, least harmful to the bacilli and especially suitable for making the final aqueous suspension.

Bacilli obtained by the chloroform method are distinctly less deeply acidfast than those in control preparations, although no more so than many of the isolated bacilli in smears of regular lepromin. It was thought that removal of them from the tissues by an oil or mixture of oils that would be miscible with acetone might obviate that effect.

Since paraffin oil is particularly effective in the protection of acidfastness of bacilli in tissue sections, search was made for a suitable mixture of that oil and one of vegetable origin. Such a mixture might perhaps restore full acidfastness to the older and more decrepit bacilli always present in lepromas, especially in those deriving from sulfone-treated cases.

Only coconut oil with 20 per cent or less of paraffin oil proved miscible with acetone, except thin cedar oil (as used for embedding) which was in short supply and rather expensive. Coconut oil, although easily made fresh and clear where coconuts are available, would not be generally available; it could be made from commercial shredded and desiccated coconut, but that would entail some effort.

The matter was not pursued, however, particularly because it was felt that the more simple preparation made by the chloroform method should first be tested clinically before trying out a preparation the antigenicity of which might possibly be enhanced artificially by the oil treatment (Freund's adjuvant principle). It might still be worth while to try out such an antigen.

PREPARATION OF THE SUSPENSION

A pooled lot of autoclaved lepromas from several patients was carefully cleansed of extraneous material, sliced thin, and mixed thoroughly by pounding in a mortar. After weighing the resulting coarse pulp, one-half of it was used to make a control lot of standard lepromin ([21]), and the other half was used for preparing the purified bacillus suspension. Similarity of the original condition of the batch of bacilli used for making the two preparations was thus assured.

The aliquot used for the suspension was, as in Dharmendra's technique, ground up in a mortar in 30 cc. of chloroform for about 10 minutes, after which what part of the chloroform had not evaporated was pipetted off to a flask through a nylon filter. This was repeated four times, until relatively few bacilli remained in the tissue pulp.

To the final volume of the chloroform suspension (80 cc. remaining

[2] Using the Boerner centrifugal filter, A. H. Thomas & Co. 5114-C, with Seitz filter pad.

of the 150 cc. of chloroform used), twice that amount (160 cc.) of acetone was added. The mixture became faintly clouded, but what part of that clouding was due to the regained opacity of the bacillus and what part—if any—was due to precipitation of some substance that was soluble in chloroform but not in acetone could not be ascertained. (However, see Addendum.)

The mixture was distributed to six 50-cc. centrifuge tubes, after which each was covered with a piece of aluminum foil, secured by rubber bands, to prevent loss by evaporation. Centrifuging at 2,000 r.p.m. for 20 minutes produced compact deposits that were fairly solid, with perfectly clear fluid above them.

From each tube all but a little of the supernatant was decanted. With a rubber-tipped glass rod each deposit was resuspended in the remaining fluid and poured into a single tube, after which each of the other tubes was rinsed by a small quantity of the clear supernatant. The total bacillary material so collected was deposited by another run in the centrifuge, and the supernatant was discarded.

The deposit could, of course, have been dried and an antigen made up by weight. It was preferred, however, to standardize the product by microscopic comparison with the lepromin to be used as the control. So, while the deposit was still wet, it was thoroughly rubbed in a quantity of phenol-saline.[3]

Smears of this concentrated product showed no blue-staining tissue particles. After dilution, standard smears were compared with similar smears of the standard lepromin. Because, as said, the bacilli appeared somewhat paler and thinner as a result of the exposure to chloroform, the final suspension was made to contain about 50 per cent more bacillary bodies than the lepromin, as accurately as could be determined by microscopic inspection.[4]

This standardization by comparison is not as simple as comparison of two lepromins. Because the suspension is a relatively pure aqueous product, without the proteins present in lepromin, the standard (3 mm.) platinum loop would pick up only a thin, flat membrane, containing much less material than the thick meniscus of lepromin that the loop would hold. It was therefore necessary to resort to the use of micropipettes, prepared from ordinary glass tubing, by means of which measured amounts (e.g., 0.01 cc.) of the different products were spread over measured areas on the slides.

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[3] Actually, as a rather thin paste made with a little phenol-saline, the deposit was transferred to a piston-type tissue grinder, or homogenizer, with a Teflon pestle (A. H. Thomas Co. No. 4288-B), and ground for some 15 minutes to aid dissemination of the compacted bacillary mass, after which it was further diluted.

[4] An essential point, not seen emphasized, is that the slides on which the preparations are made must be perfectly greaseless, the surface receptive of water so that it spreads. This we accomplish by vigorous rubbing with a water-wet clean towel. (Shepard, in an article in this issue of THE JOURNAL, speaks of "slides that have been cleaned by vigorous rubbing with an alcohol-moistened towel," with which the same effect can be obtained.)

Experiments were done with serum protein, and with thin gelatin solutions, to insure adherence of the bacilli of the purified suspension to the slide. That maneuver, however, proved unsatisfactory; and, if care was taken to avoid undue turbulence in the staining solutions, it was quite unnecessary.

The finished suspension, a thinly-clouded product, was found on microscopic examination to contain mostly free-lying bacilli, with many loose clumps but no dense globar masses because they had been dispersed by the chloroform. No solid tissue particles were to be seen. As had been done with the lepromin, it was then put up in 15 cc. vials and autoclaved. This preparation, presumably, resembled to some extent the one made by Lowe. The results of tests with the preparation are to be reported separately.

SUMMARY

The various methods that have been employed for obtaining tissue-free suspensions of leprosy bacilli are reviewed briefly. Most of them were eliminated from consideration for practical purposes, because they do not meet the primary requirements of simplicity of technique or—and especially—of economy of the leproma material.

After experimentation it was found most practical to harvest the bacilli from the pulped leproma tissue with chloroform, as in the first step of preparing Dharmendra's antigen, and then to dilute the pooled chloroform extract with two times its volume of acetone, after which the bacillary bodies are deposited by centrifuging and resuspended in phenol-saline.

The bacilli so obtained are affected to some degree by the chloroform, being distinctly paler in color and of thinner appearance after Ziehl-Neelsen staining than those in the standard lepromin made from an aliquot of the same starting material. Consequently, the final suspension was made up with approximately 50 per cent more bacilli than the lepromin made for use as the control in clinical tests.

RESUMEN

Se repasan brevemente los varios métodos empleados para obtener suspensiones histoprivas de bacilos leprosa. La mayor parte de ellos quedaron eliminadas de consideración para fines prácticos por no cumplir los requisitos primordiales de sencillez de la técnica o—y en particular—de economía del material lepromatoso.

Después de experimentar, resultó lo más práctico recoger con cloroformo los bacilos del tejido del leproma hecho pulpa, lo mismo que en el primer tiempo de la preparación del antígeno de Dharmendra, y diluir luego el extracto clorofórmico combinado con un volumen doble de acetona, después de lo cual se despositan los cuerpos bacilares por centrifugación y se resuspenden en solución salina-fenol.

Los bacilos obtenidos en esa forma son algo afectados por el cloroformo, teniendo evidentemente color más pálido y aspecto más delgado después de la coloración de Ziehl-Neelsen que los de la lepromina estándard preparada de un porción alícuota del mismo material inicial. Por consiguiente, se preparó la suspensión final aproximadamente con 50

por ciento más bacilos que la lepromina elaborada para empleo como testigo en las pruebas clínicas.

RESUMÉ

On passe brièvement en revue les différentes méthodes qui ont été utilisées pour obtenir des suspensions de bacilles de la lèpre dépourvues d'éléments tissulaires. La plupart de ces méthodes n'ont pas été prises en considération pour leur application pratique, car elles ne répondent pas aux impératifs promordiux de cimplicité technique ou, surtout, d'utilisation parcimonieuse du matériel lépromateux.

Aprés essai, il a été trouvé que la méthode la plus pratique était d'extraire au chloroforme les bacilles de la pulpe de tissu lépromateux, comme dans le premier stade de la préparation de l'antigène de Dharmendra, et ensuite de diluer l'ensemble des extraits chloroformés par deux fois son volume d'acétone, après quoi les corps bacillaires sont recueillis par centrifugation et suspendus á nouveau dans une solution physiologique phénolée.

Les bacilles ainsi obtenus sont modifié dans une certaine mesure par le chloroforme. Après coloration de Ziehl-Neelsen, leur couleur est nettement plus pâle et leur apparence plus mince qu'il n'est observé dans la lépromine standard préparée à partir du même matériel de départ. En conséquence, la suspension finale a été constituée d'approximativement 50% plus de bacilles que la lépromine destinée au contrôle dans les tests cliniques.

ADDENDUM: NILE-BLUE STAINING OF SMEARS

Recently Mori, Kosaka and Ito ([15]) have called attention to the fact that apparently pure bacillary suspensions (of the murine bacillus) prepared by trypsin digestion of the tissue elements are not actually free from residual material. Although smears by the Ziehl-Neelsen method and counterstained with methylene blue seemed quite clean, they nevertheless still showed a considerable amount of contamination when nile blue was used for the counterstain. This counterstain consists of 2 parts of a saturated aqueous solution of nile blue mixed with 1 part of 95 per cent ethanol.

Although the chloroform-acetone method of preparing the purified bacillus suspension in no way resembles the trypsin method, the nile-blue counterstain has been tried out on the product. From a vial of the PBS which had stood in the refrigerator for nearly five years, smears were made after thorough shaking.

The methylene-blue showed an abundance of bacilli of a rather dull reddish color, many free-lying but mostly agglutinated in small to large loose clumps and skeins. The smear was all but perfectly free from any blue-staining material, and what little there was seemed to be of extraneous origin.

The nile-blue smear, on the other hand, had a distinctly different appearance. Under the high-dry objective, the bacillary clumps were considerably darker in color. With the oil-immersion objective most of the larger loose clumps were seen to contain a faintly-staining matrix material—if "matrix" is an appropriate term for a substance so tenuous. Furthermore, the background of the smear also was faintly bluish, although not enough so to be evident to the naked eye.

Thus the purified bacillus suspension appears—at present, at least —to contain a small amount of some sort of contaminating material, demonstrable by nile-blue but not by methylene blue, which apparently remained after the chloroform-acetone treatment. Presumably this consists of an acetone-insoluble substance that had been precipitated from the perfectly clear chloroform suspension, contributing to the slight clouding seen when the acetone was added. Whatever its chemical nature (phospholipid?), it seems unlikely that it could have held any effect on the Mitsuda reactions caused by the suspension.

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