THE LABORATORY DIAGNOSIS OF LEPROSY

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It is now more than three-quarters of a century since Armauer Hansen, in 1874, first made public his finding of the leprosy bacillus. Hansen was seeking an extrinsic agent, a contagia, as the cause of leprosy, in order to remove this malady from the category of hereditary diseases, where it has been so firmly intrenched by the authority of Danielssen and Boeck in 1847. He was convinced, on the basis of his investigations of syphilis, with which he was prone to compare leprosy, that leprosy was a contagious disease. In considering fixed films of leprous tissue examined microscopically, he states in the original report, "In some cells one finds bunches of rods and some cells are, as it were, packed with them." Having made this preliminary announcement, Hansen carried on with sedulous zeal his study of the nature and distribution of the rods, and proved that, in lepromatous leprosy, they were to be found in all typical nodules, in the spleen, the liver, and particularly the lymph nodes. They were never present in healthy individuals or in patients with other diseases. He therefore concluded that these rods were the cause of leprosy. Coming as it did during the prelude to the acceptance of the germ nature of disease, his belief was shared by a relatively small number of leprologists.

In connection with the discovery of the tubercle bacillus by Koch in 1882, a staining technique was developed which differentiated Hansen's bacillus and Koch's bacillus from other microorganisms. The stain could be applied directly to tissue films and the two germs detected if present in sufficient numbers. Thus, to the clinical signs and symptoms, there was now added the microscopic search for Hansen's bacillus in the diagnosis of leprosy. Koch, however, promptly discerned the limitations of identifying an organism as the etiological agent in a suspected infectious disease by differential staining, because of possible contamination with extraneous saprophytic forms. He insisted, before any such relationship be advanced, that the germ seen in the stained film be grown in pure culture on artificial media, and that the inoculation of a susceptible laboratory animal with a suspension of the pure culture induce an infection identical with the natural occurring disease. These steps, later designated as "Koch's Postulates," were implemented and became the dogma to be fulfilled before sponsoring a specific germ as the cause of a specific disease.

With the passage of time and the increases in knowledge of changes occurring in the host as a result of infection, two additional diagnostic approaches were developed: (1) serological reactions employing the serum of the patient in such tests as agglutination, precipitation, and complement-fixation, noting particularly increases or decreases in the titer of the serum in successive samples; and (2) immunological reactions, *i.e.*, the failure to induce disease in specifically immunized animals by the injection of suspensions of homologous virulent organisms or their toxic products. Un-

fortunately, these advances in laboratory techniques, which are routinely employed today in the diagnosis of most of the infectious diseases, are not applicable to leprosy. Cultures of Hansen's bacillus on artificial media are obtained only with the greatest of difficulty; purposeful attempts to transmit leprosy to laboratory animals, including man, have been uniformly negative; specific serological reactions have been most unsatisfactory, and, with the exception of the lepromin reaction, which will be covered by one of the other writers in this monograph, the standard immunological tests are of no aid in diagnosis or prognosis in Hansen's disease.

Leprosy stands unique in the field of infectious diseases in that the laboratory is able to aid the clinician in arriving at a diagnosis only in so far as the presence or absence of Hansen's bacillus can be detected by microscopic methods. Nevertheless, once a diagnosis of leprosy has been established, the microscopic method is of inestimable value in the clinical classification of cases, and it is the only method for the unequivocal evaluation of chemotherapeutic substances at present.

Today, more than at any time since the development of the Ziehl-Neelsen stain, a meticulous bacteriological examination in leprosy is essential. The pronounced clinical improvement which regularly follows the therapeutic use of the sulfone compounds is not paralleled by the disappearance of Hansen's bacillus from the tissues. As a result, many patients would be prematurely removed from sulfone therapy on clinical grounds alone, to enter society and become foci for the spread of leprosy, were it not for this one bacteriological control.

A number of excellent descriptions of the techniques for the bacteriological examination have been published. These have been liberally drawn upon to augment my own experiences in the preparation of the following material. No citation of references is given, since it would express inadequately my debt to those workers with whom I have been associated in the field. In general, emphasis is directed to the importance for a rigid procedure in the obtaining of the specimens. Confidence is placed in the ability of the laboratory worker to proceed with the staining and subsequent microscopic examination once the films have been made. Too often, this is misplaced confidence. Too frequently, the clinician prepares the films, sends them to the laboratory, and, at a later date, reads and attempts to interpret the reports. It is eminently more satisfactory for the individual in charge of the laboratory to assist or at least be present when the patient is examined and to discuss the laboratory findings in conference.

The techniques of the bacteriological examination may be conveniently grouped under four headings, the selection of sites for examination, the obtaining of specimens and making of films, fixation and staining, and microscopic examination and recording of data.

In the selection of areas for examination, much depends upon the experience of the investigator. After routine specimens have been taken from the edges of the earlobes, the forehead just above the eyebrows, the cheeks, the chin, and the elbows, the patient should strip and be placed in a good light. Films are then made from the advancing edges of obvious

nodules and the fingers are passed over the skin surface, especially that of the buttocks and upper and lower limbs. If nodules or thickenings of the skin are detected, the edges of these areas should be sampled. It is very desirable to include a film from what appears to be normal skin to serve as a control.

The question of how many specimens should be taken from each patient always arises. It is the consensus of competent leprologists that six to eight films, the number that can be conveniently placed on one slide, is adequate to obtain a general impression of the richness of the infection. This number is probably the minimum in confirming a clinical diagnosis. Once the diagnosis has been made and the patient is under therapy, sixteen to twenty-four films will enable a better assessment of the progress of the treatment. It should be recalled, *ad nauseam*, that a thorough study of a few carefully prepared specimens is of greater value than a superficial examination of many carelessly made preparations.

The nose is examined with the aid of a head mirror and a nasal speculum and material taken from suspicious areas. The constant reference to lesions in the nose and their importance in bacteriological studies was not warranted in presulfone days, since films from the nose were rarely if ever positive in the absence of positive skin films. As a result of the introduction of these drugs, however, it has been observed that the nasal smears become negative earlier in the course of treatment than skin smears taken from different parts of the body, thus giving unusual importance to the nasal examinations.

There is no cavil as to the best technique for obtaining specimens and making smears. The older methods of excising a piece of tissue with a pair of curved scissors or placing clamps on the skin and subsequently gathering serum or lymph from the isolated area have been entirely superseded by the "scraped-incision" variety of the so-called "slit method," carried out as follows: The area to be examined is thoroughly cleansed by rubbing vigorously with a small pledget of cotton saturated with alcohol. Tincture of iodine is unquestionably the reagent of choice for this purpose. Unfortunately, the stoppers of the containers are often carelessly handled, permitting the evaporation of the solvent and the concentration of the iodine. Application of the residue to the skin causes destruction of the tissue. One of the objectives of the cleansing of the skin is to remove any saprophytic acid-fast bacilli which might lead to false positive reactions. It should be done thoroughly, therefore.

A fold of skin containing the lesion is compressed between the thumb and forefinger of one hand and a short incision two to three mm. in depth is made with a scalpel or Gem-type safety razor blade. The cutting instrument is then rotated perpendicular to the wall of the incision and scrapings made, particularly of the corium, to obtain tissue pulp. An excess of blood is to be avoided. The scrapings are transferred to a clean new slide and spread uniformly over a small area. As indicated above, multiple smears are prepared on one slide and identified, using a wax pencil. To avoid contamination of subsequent smears, the instrument used for obtaining the

specimens should be thoroughly wiped between each sampling with fresh cotton soaked in alcohol.

Nasal specimens are taken from suspicious areas after the mucosa has been anaesthesized with cocaine and mucous or other surface material has been wiped away. A short nichrome wire (the regular inoculating needle of the bacteriologist), bent and hammered at the end to form a sharp scraper and then mounted in an aluminum holder, is very suitable for this purpose. It has the additional advantage that it may be repeatedly sterilized in the flame, thus avoiding the remotest possibility of introducing organisms from positive films to negative ones. The sample should be removed by scraping deep enough into the lesions or normal tissue to cause slight bleeding. All films are allowed to air dry and are stored temporarily in regular slide boxes to prevent contamination with air-borne acid-fast organisms, which may be fairly commonplace.

Fixation and staining of the specimens should be carried out promptly. In an era when every effort is being made to standardize procedures, it is hardly in keeping with good technique to fix films empirically by passage of the slide through the flame until the temperature of the slide can no longer be tolerated when pressed on the back of the hand. A glass or metal plate supported over a boiling water bath serves ideally for fixing and staining. Metal bars with enclosed electrical heating elements are also available for this purpose. The slides are placed on the plate and fixed for two minutes, then covered with fresh Ziehl-Neelsen solution (0.5 per cent basic fuchsin, 5 per cent phenol, 10 per cent ethyl alcohol in distilled water) and stained thirty seconds. The slide is rinsed in water, the films decolorized in 3 per cent hydrochloric or 5 per cent sulfuric acid and in 95 per cent alcohol, rinsed in water, counterstained by flooding with an aqueous solution of methylene blue for fifteen seconds, rinsed in water, and the slide then supported on edge to dry.

The use of auramine as an acid-fast stain for the examination of tubercle bacilli in fluorescent light, introduced by Hagemann (1938, Munch. Med. Wschr. **85**: 1066.), is of great value in the examination of specimens from patients with leprosy. This technique permits the use of the low-power lens with a pronounced increase in the size of the microscopic field. The details will not be given, since special equipment is required.

The examination of the slides should be carried out in an orderly manner. A gross inspection of the films is made, noting the general appearance of the stains, the amount of material present, and the uniformity of distribution. Turning to the microscope, which is an instrument of precision, a mechanical stage is desirable, and a meticulously clean lens system is essential. The film of normal tissue is regularly examined first as a control. A preliminary survey of the films with the low power lens ($\pm 100\times$) will often detect large clumps of acid-fast organisms and serve to guide the subsequent examination with the oil immersion system. Several commercial preparations are now available which are superior to cedar oil as an immersion fluid.

The recognition of Hansen's bacillus on the basis of morphology, staining

reaction, and post-fission grouping is not simple. The features to be noted with the oil-immersion lens are: the presence or absence of acid-fast bacilli; the appearance of the stained cells, whether the rod is uniformly red or composed of highly refractive granules, giving a beaded appearance to the cell; the distribution of singles and clumps of two or more bacilli; the groupings in characteristic cigar-shaped bundles; the position of the bacilli, whether free or intracellular; and the presence of non-acid-fast forms resembling diphtheroids and so-called ghost-cells. A slide should not be declared negative until at least 200 fields have been examined.

It is well to pause here and consider the changes in Hansen's bacillus as a result of sulfone therapy. The action of these compounds is believed to be bacteriostatic and not bactericidal. Within a few weeks after the initiation of treatment, the organisms in films from the nose become less acid-fast and stain irregularly. Granulation is more conspicuous, the granules appear to be larger and take a deeper stain. The ratio of ghost-cells and diphtheroids increases and, within a few months, the nose becomes negative. Skin smears from the same patients may continue to be positive for as long as four or five years, the number of organisms becoming progressively smaller, with the same changes in the staining reactions as observed in nasal smears. The high relapse rate in negative patients who have discontinued drug administration would suggest that a more thorough bacteriological study, including material aspirated from the lymph glands, would have detected the presence of germs, and therapy would have been continued. An additional observation has been noted in connection with the bacteria in globi. Following sulfone therapy, the organisms gradually lose their uniform staining quality. Granulation of the cells occurs in a progressive manner and the contents of the globus become amorphous, exhibiting a pale pink color. This was noted in presulfone days but seems to be more conspicuous since the introduction of these drugs. It may be the result of autolysis or the defensive mechanism of the host because of the bacteriostatic action of the sulfones.

The various staining methods devised to distinguish between living and dead cells, as well as the methods designed to differentiate Hansen's bacillus from Koch's bacillus, have been exhaustively studied and have been without merit in the hands of the author.

The number of bacilli varies in different patients from none or one or two suspicious forms to smears that appear to be films of acid-fast organisms only. It is extremely desirable to have some method for indicating the frequency of the bacilli or degree of positivity in each smear. Unfortunately, the work of Wassermann in reporting the results of the positive complement-fixation reaction in syphilis in terms of 1+, 2+, 3+, and 4+ has established a pattern for all subsequent designations in bacteriology. In keeping with this system, the incidence of acid-fast bacilli in leprosy are placed under five categories: negative or −, when no typical forms are detected; 1+, few bacilli, singles and occasional clumps; 2+, moderate, ignoring single bacilli, a group or groups of bacilli are found on the average in 5 per cent of the fields; 3+, numerous, a group or groups of bacilli are found in 15–20 per cent of the fields, and 4+, rich, every field with many

globi and intracellular forms. The trained investigator, accustomed to routine laboratory work, arbitrarily and subconsciously arranges the films under these headings with an unusual degree of regularity. The designations of different workers will vary within certain limits, but it is gratifying to find a real thread of consistency in the reports of individuals from widely separated laboratories.

A further refinement, namely the obtaining of the "Bacillary Index," has been introduced in the recording of data. The need for such a system has been stimulated by the favorable showing with sulfone therapy. The B.I. represents the average of the values assigned the films taken from a patient, and, when calculated in successive examinations, is sponsored as a yard-stick for assessing the value of treatment. This system of recording has not as yet been widely accepted.

No reference has been made to the histological findings or the distribution of Hansen's bacillus in the different clinical forms of leprosy. This will be fully covered in the paper devoted to pathology. A discussion of the histamine reaction has been purposefully omitted since it can hardly be considered a laboratory procedure. The same attitude is taken toward the use of tuberculin or lepromin by certain clinicians to reactivate bacteria-free suspicious lesions.

Summary. The discovery of Hansen's bacillus, *Mycobacterium leprae*, and its acceptance as the etiological agent of leprosy, placed this disease among the first in which laboratory techniques from the new science of bacteriology were of unequivocal aid in establishing a diagnosis. Unfortunately, today, as in the beginning, the only laboratory procedure available is the microscopic examination of stained specimens for the presence of organisms which, on the basis of morphology, staining reactions, and post-fission grouping, are typical of Hansen's bacillus. Since the bacteriological examination is so essential for diagnosis, classification, and evaluation of the results of treatment, every safeguard must be employed to ensure maximum efficiency. Additional controls, such as cultural studies on special media, and guinea pig injections, to eliminate the question of the presence of saprophytic acid-fast organisms, tubercle and pseudotubercle bacilli, should be employed.

Research will uncover new laboratory methods in leprosy, but, until these are available, full use must be made of the bacteriological examination, lest it fall in disfavor and progress in leprosy be based on empiricism.