REPORT OF THE PANEL ON BACTERIOLOGY AND IMMUNOLOGY[1]

BACTERIOLOGY[2]

This report emphasizes: (a) investigation of the cytology and metabolism of *M. leprae* recovered directly from lesions and (b) its cultivation independently of animal cells..

Cytology and histochemistry.—Successful cultivation of *M. leprae* will not decrease the importance of cytologic examination of leprosy bacilli obtained directly from lesions.

International conventions are needed to ensure (a) uniform methods for obtaining samples and preparing smears, and for staining and differentiation; (b) a logarithmic or arithmetic scale for expressing concentrations of bacilli, and (c) classification and interpretation of the morphologic features in *M. leprae*. The immediate goal should be to estimate correctly the proportion of solid-staining forms.

In routine examinations in lepromatous, borderline and dimorphous cases work can be saved by recalling that ear lobes, nasal mucosa and margins of palpable or active lesions are the sites most frequently and strongly positive, and the last sites to become negative during therapy. Elimination of nasal scrapes is recommended.

Microscopic work can be reduced by greater use of low power objectives. The Committee recommends certain modifications of Muir's system of grading. The oil immersion objective is used to count bacilli when they are rare. The low power (100× mag.) is particularly useful in strongly positive cases. The bacteriologic index is the average of the values assigned to individual films at each examination.

Difficulties in simultaneous metabolic study of parasite and host include (a) distinction between the specific activities of each, (b) the fact that microorganisms grown *in vivo* do not utilize exactly the same systems they use *in vitro,* and (c) the limited availability of microbes in most infections. Advantage should be taken of leprosy to investigate relationships between cytologic and histochemical properties of *M. leprae;* facts can be developed by simultaneous studies of other mycobacterial cells that are growing, resting or blocked by chemotherapy.

Biochemistry and metabolism.—In the investigation of the biochemistry and metabolism of rat and human leprosy bacilli specific functions can be analyzed without the complexities of growth. One goal is to identify key systems that require supplementation to permit

---

[1]The Panel was composed as follows: Dr. J. H. Hanks, *chairman*, Dr. J. M. M. Fernández, *secretary*, and Drs. J. O. Almeida, M. P. Azevedo, J. E. Cardama, R. S. Guinto, S. W. A. Kuper, E. Montestrue, J. H. S. Pettit, A. Rotberg, C. Silva, N. Souza Campos, K. Yanagisawa, Y. Yoshie, *members*.

[2]Sub-committee on Bacteriology: Dr. J. H. Hanks, *chairman*, and Drs. J. H. S. Pettit, Y. Yoshie.

growth of the organism *in vitro;* another is to learn the manner in which existing systems permit growth within hosts.

Enzymes liberated from cell-free extracts of rat and human leprosy bacilli have provided evidence of conventional components in the glycolytic, hexosemonophosphate, and citric acid cycle systems. Several lines of evidence direct interest toward problems in terminal respiration. Nevertheless, biochemical investigations have been handicapped in the same manner as bacteriologic studies. They have not yielded clues on factors permitting *M. leprae* to expand the key systems required for growth *in vitro.*

The cultivation problem.—The central importance of *in vitro* cultivation of *M. leprae* is widely recognized. Factor-requiring and noncultivated mycobacteria in animal hosts in every country afford opportunity for exploring the unconventional conditions and factors required by host-adapted mycobacteria. Investigation of special problems in factor-requiring and presently uncultivated species may improve methods for growth of conventional species and also provide models of the types of deficiency to be anticipated in *M. leprae.*

Cultivable species: The number and variety of strains now known to be associated with the respiratory tract and those recovered year by year from leprosy indicate clearly that potentially parasitic or pathogenic mycobacteria are ubiquitous. Important questions include: (a) Are such strains recovered from leprosy patients because of residence in ulcers, or when spread elsewhere on the skin? (b) Are they similar to strains known to cause cutaneous ulcers or to those associated with the respiratory tract? (c) Can they be eliminated from biopsies for *M. leprae* by skin sterilization? (d) May they at times be found within nonulcerated tissues after skin sterilization? (e) Are there patients in whom these strains induce or complicate reactional states? Cultivable strains of mycobacteria from body surfaces are of genuine interest to microbiology and immunology. Several types of mycobacteria persist together during subculture of freshly isolated "strains." Repeated plating, therefore, is necessary.

Noncultivated species: Nearly 100 years of unsuccessful effort to propagate *M. leprae* on bacteriologic media, and more recently in cell cultures, have failed to yield clues on the character of obstacles to be overcome.

1. In selecting a model yielding both cultivation and biochemical data, saprophytes, tubercle bacilli, and other independently growing strains are largely ruled out, while the factor-requiring *M. johnei* and the woodcock bacilli qualify. On withholding the factor, they are converted to noncultivable states and may become counterparts of *M. leprae* in several respects. We also should know more of factor and growth requirements of other noncultivated mycobacteria. These may show other and more specialized requirements.

2. Problems for profitable study include: (a) biochemical and metabolic differences between growth-competent and incompetent cells, (b) definition of systems rehabilitated by addition of factors essential for growth, (c) materials and conditions for optimal utilization of growth factors, (d) comparative merits of host cells and of other microbes as a source of essentials.

Criteria for *M. leprae:* Before concluding that one has cultivated *M. leprae* the following criteria should be met: (a) significant, transplantable growth should occur through use of a new principle, factor(s) or condition(s); (b) cultures should be obtained from all lepromatous patients and nearly all tuberculoid cases; (c) distinctive nutritional and metabolic properties should be stable; (d) serologic differentiation from other species should be definite; and (e) suspensions adjusted to equivalence with control lepromin, and simultaneous skin tests in the same individuals, should be employed. In experiments in cell cultures and animals, the requirements are: significant increases in bacterial counts, serial transmissibility, noncultivability, and points *d* and *e* above.

IMMUNOLOGY[4,5]

The following facts and views are outlined as basic to current understanding of the pathogenesis and immunology of leprosy.

Genetic background in man.—All animals make effective response to thousands of antigens. Genetic factors must be considered in order to explain the exceptionally wide range of resistances and cutaneous reactivities in leprosy in man. Individuals in any population tend to fall into one of three groups: 1. Capable or average responders,[6] the majority of whom are, or become, resistant. 2. Slow responders, who are susceptible to leprosy, and develop the nonlepromatous forms. Lesions tend toward self-healing. Cutaneous reactivity often develops slowly and in modest degree. 3. Poor responders, in a small minority of whom leprosy is persistent and progressive. Capacities for resistance tend to remain at insignificant levels.

---

[4]Sub-committee on Immunology: Dr. J. H. Hanks, *chairman*, and Drs. M. P. Azevedo, J. E. Cardama, J. M. M. Fernández, R. S. Guinto, S. W. A. Kuper, E. Montestruc, A. Rothberg, K. Yanagisawa.

[5]The Subcommittee on Serology, a subcommittee of this Panel, sponsored a Work Conference on Serology of Leprosy during the Congress, in cooperation with the Pan-American Health Organization. The report of this subcommittee was not available at the time of printing this issue of the JOURNAL. It will appear, however, in the complete Transactions of the VIIIth International Congress of Leprology. Reports from the Work Conference can be obtained in the meantime from the Pan-American Health Organization, 1501 New Hampshire Avenue, N.W., Washington, D. C. 20036 (Ref.: Res 63.3, 27 November 1963).

[6]In order to group a multiplicity of phenomena in three classes, the term chosen cannot be technical or specific. "Response" seems to cover a series of concepts: proneness to infection, performance during recognized disease, ability of cells to destroy *M. leprae*, cutaneous reactivity, etc.

Basis of acquired resistance.—Native and acquired resistance to mycobacterial infections have not been shown to be due to, or associated with, antibodies or antibody production. Native resistance may depend in part upon factors unfavorable to reproduction of *M. leprae;* it is rapidly fortified by acquired resistance. Acquired resistance depends primarily on improvement in the natural capacities of mesenchymal cells to digest mycobacterial cell walls and hydrolyze their protein components. Since the fundamental reactions for the destruction of microbes are hydrolytic, they are less specific than antibody production and serologic reactions but more primitive and fundamental.

Cutaneous reactivity.—The connection between cutaneous reactivity and resistance rests on the fact that microbial cell walls are naturally toxic and digestible in animal mesenchyme cells. Rates of destruction are originally higher and more readily enhanced in resistant than susceptible families of animals. The "immunized" animal or person, by destroying bacterial walls more rapidly, liberates toxic components at a greater rate. The degree of "sensitivity" will depend on hydrolysis rates, inherent toxicity of bacterial derivatives, and differing susceptibilities to damage of tissue cells in various animal species. As an example, killed tubercle bacilli are destroyed more rapidly than *M. leprae,* while the soluble derivatives appear to be more toxic. The end result is that tubercle bacilli stimulate stronger "sensitizations" than those induced by *M. leprae.*

Natural causes of cutaneous reactivity.—In general populations stimulations occur haphazardly. The per cent of positive reactors is related importantly to age. Many previously "unknown causes" of reactivity can now be identified. The list of naturally encountered infections and/or carrier states now includes: (a) pulmonary infections caused by *M. kansasi,* the avian-Battey complex, and other unclassified strains from Runyon's groups I-IV; (b) cutaneous infections and ulcers caused by *M. ulcerans, M. balnei,* the Kakerifu Kasongo strains in the Congo, and probably also strains of the types repeatedly isolated during efforts to cultivate *M. leprae.*

Intact mycobacteria are antigenic. A single test with 5 TU of PPD has a "booster" effect in mild and moderate tuberculin reactors. It would be unwise therefore to suppose that soluble components of *M. leprae* are incapable of enhancing cutaneous reactivity.

Epidemiologic implications of cutaneous reactivity.—Leprosy infections do not create strong sensitizations. There is no outstanding tuberculin-type reactivity by which the spread of *M. leprae* through general populations can be detected, but the use of leprolins from washed leprosy bacilli offers a possibility of finding persons who react more strongly to derivatives of *M. leprae* than to similar preparations from tubercle bacilli and other related species. The Mitsuda reaction may be used to assay the potential for resistance in populations. For

this purpose, one is not concerned that cutaneous reactivity may have been created through cross-stimulations by other mycobacterial antigens.

Immunization.—1. BCG and other mycobacteria stimulate Mitsuda positiveness, but not in all persons. They fail to affect "poor responders," i.e., those incapable of adequate response to *M. leprae* itself. 2. Available stimulators, e.g., BCG plus small amounts of specific antigen in the form of killed *M. leprae*, may prevent or ameliorate disease among "slow responders." 3. For the majority of persons, the "capable" responders, early stimulation of Mitsuda reactivity or resistance is not necessary.

Immunizing components in mycobactria.—(1) Cell walls are inherently toxic in normal animals. The tuberculin-type reactivity they induce is relatively nonspecific. Resistance is increased. (2) Soluble components liberated by rupture of cells are relatively nontoxic. They do not by themselves induce appreciable tuberculin-type reactivity, but have a potent and relatively specific immunizing effect. In immunized animals both fractions elicit tuberculin-type skin reactions.

Summary of concepts in the foregoing.—1. Preventive immunization, chemoprophylaxis, and therapy must be designed to benefit that small segment of humanity poorly equipped genetically to deal with *M. leprae*. 2. Genetic factors and prior exposure to specific or cross-reacting antigens are major determinants of resistance and of cutaneous reactivities to soluble products or intact bacilli. 3. The prognostic significance of the Mitsuda reaction depends upon innate capacities for intracellular digestion of the toxic walls and proteins of *M. leprae*. 4. For the majority of persons in a population, immunization against leprosy is not necessary.

Assay and interpretation of cutaneous reactivities.—Study of cutaneous reactivities to heat-killed *M. leprae* and its soluble products has been a major tool for investigating immunology and resistance in leprosy.

(1) The Fernández reaction: The Panel subscribes to the reading and interpretation of the Fernández reaction as defined in the Reports of the Madrid (1953) and Tokyo (1958) Congresses.

(2) The Mitsuda reaction: Mitsuda reactions are induced by the intradermal injection of lepromin (*M. leprae* in heated leproma suspensions) or washed *M. leprae*. They are analogous to mild or slowly developing Koch reactions to tubercle bacilli. Readings of the Mitsuda reaction assay rates of destruction of mycobacterial cells and should be spaced to yield data that can be plotted as time-profile curves. The practical significance of these points is illustrated by more detailed consideration of the Fernández reaction, and the Wade and Olmos Castro phenomena.

Standards and reading of the Mitsuda reaction: The Panel makes three new recommendations: 1. The provisional standard for lepromin should contain 160 million bacilli per cc. 2. Mitsuda reactions of less than 5 mm. average diameter are of doubtful significance. 3. Careful comparisons should be made between reactions to the provisional standard lepromin and a 1:8 dilution of the same, preferably in the same individuals. It may be possible to eliminate nonspecific reactions due to tissue components and to expand the existing supplies of lepromin by 8 times.

It is reasonable to regard positive Mitsuda reactions as expressions of resistance to leprosy, confidence being proportional to the degree of positivity. Doubtful reactors should be reexamined after 6 to 8 weeks. Ulcerations should be recorded; they are not retained as a special grade of reaction, since they may be created by itching or scratching.

LEPROMIN

On the basis of efficient recovery of bacilli and relative convenience, Wade's modification of the Mitsuda-Hayashi method is preferred. Recent studies, however, demonstrate that two quite different types of result arise from current efforts to prepare lepromin. In one study, 16 lepromins (prepared in 10 separate laboratories) differed by 800 times in bacillary content. Successive lots from some laboratories were found to differ by 100 or 200 times. Hence it must be questioned if existing data on Mitsuda reactivity are comparable from place to place and time to time.

Another group of workers, on the other hand, reported that: (1) lepromins made in two different countries agree closely in bacillary content and skin test potency; (2) highly reproducible lepromins can be made by pooling of selected lepromas and by minimizing discrepancies in tissue dilution; (3) biologic standardization can be conducted in guinea-pigs more conveniently than in man; (4) lepromin is antigenic in man; and (5) tissue components sometimes contribute an element of nonspecificity when full-strength lepromins (tissue 3-5%) are employed in testing.

The need to sharpen the prognostic significance of the Mitsuda reaction has been evident for some years. Tabulations of the prognostic value of Mitsuda reactions have revealed that 2+ and 3+ reactions have great significance, while 1+ (3-5 mm.) reactions do not.

New recommendations are made by the Panel with respect to the size of 1+ Mitsuda reactions. The table below sets forth percentages estimated by Yanagisawa and associates as false-positive reactions when the 1+ grade of response was judged by the three existing criteria for reading.

Criteria for 1+ reactions			Per cent false positives among 1+ reactions	Per cent Mitsuda positives among nonlepromatous cases
Madrid	1953	3 mm.	20.0	94-99 average 97.0
WHO	1958	5 mm.	5.0	78-93 average 86.0
Japan	1955	7 mm.	1.5	65-80 average 73.0

While a recommendation of 7 mm. for 1+ would eliminate nearly all the false positives, it would eliminate also some 13 per cent of positive reactors among nonlepromatous cases of leprosy (see table). Likewise it would have a significant effect on judgments on percentages of potentially resistant persons during population surveys. The 5 mm., or WHO criterion, therefore is recommended.

Recommendations are made also for *diluted lepromin*: Economy, though often emphasized, is less important than improvement of significance without undue loss of sensitivity. In observations by Guinto and Wade on lepromins diluted 1:10 the slope of the curve relating the per cent of positive reactors to the dilution of lepromin indicated that differences would disappear if the lepromins were diluted 1:7 or 1:8 and if 3 mm. were regarded as 1+. Thus, with suitable change in criterion for positivity, the tissue components in the lepromins used could be diluted approximately 7.5 times with no loss of positive reactors. Dilutions of 1:20 would cause excessive loss of sensitivity.

In similar investigations Yanagisawa and associates studied reaction sizes considered significant when lepromins are diluted to reduce the occurrence of nonspecific reactions. With two types of lepromin it was necessary to use a 1:8 dilution to eliminate nonspecific reactions. If 5 mm. is taken as the diameter of 1+ Mitsuda reactions, a 1:8 dilution of lepromin might be expected to cut the area of reaction from 19 mm^2 to 6 mm^2, which yields an expected diameter of 2.8 mm. This means that 3 mm. reactions could be regarded as 1+, which is in agreement with the conclusion derived from the data of Guinto and Wade.

Interest has been aroused in the use of "depot" lepromin, i.e., dilute lepromin in an oily medium, administered with a tuberculin syringe or multiple puncture apparatus. The oily adjuvant conserves bacilli at the site of injection over long periods and releases antigens so gradually that these injections may be used to indicate if subsequent conversions to reactivity occur.

From data in foregoing sections it is evident that the most specific and reliable Mitsuda reactions should be induced by leprosy bacilli not altered during purification. Dharmendra-type antigens and bacilli purified by drastic enzymatic methods are universally reported to induce fewer and less persistent reactions than those obtained with Mitsuda-Hayashi-Wade type lepromins. At present, in spite of many efforts, preparation of purified suspensions remains unsatisfactory.

The results of these investigations fall into three categories:

1. If advantage is taken of the properties of chloroform (great affinity for mycobacteria and high density), relatively pure bacilli are obtained, but acidfastness is seriously impaired, cell walls are weakened, and the adjuvant effects of lipopolysaccharides are removed. As a result, these preparations elicit less frequent and less persistent reactions than those induced by lepromin.

2. Similar limitations are caused by drastic enzyme or bile treatment.

3. Less drastic procedures yielding high-quality bacilli and persistent skin reactions, are too complicated to be recommended. Purification by salt flotation and sedimentation in alcohol are inadequate and recovery of bacilli is inefficient.

While the practical problem of skin test reagents may perhaps be solved by dilution or "depot-type" preparations, there is urgent need for pure bacilli for preparation of leprolins and other immunologic and serologic purposes. This topic deserves intensive study.

SUMMARY ON IMMUNOLOGIC METHODS AND MATERIALS

1. Assay of cutaneous reactivity depends upon: (a) the Fernández reaction to solubilized proteins of *M. leprae,* as an indication of prior effective experience with related mycobacterial antigens, and (b) the prognostically significant Mitsuda reaction as a measure of rates at which cells of *M. leprae* can be destroyed. To gain maximal information on the latter point, the Mitsuda reaction must be read on several occasions over periods that may be as long as 8 weeks.

2. As a provisional standard it is recommended that lepromin should contain 160×10^6 bacilli per cc. in the presence of 3-5 per cent tissue components.

3. Given approximately 160×10^6 bacilli/cc. of lepromin (in accordance with the recommended provisional standard), problems created by nonspecific response to tissue components probably can be reduced by new decisions regarding the size of a significant reaction to lepromin 1:8 or by "depot" lepromins in oily adjuvants.

4. There is as yet no satisfactory solution to the difficulties involved in efficient preparation of washed suspensions of *M. leprae* for serologic work, for use as purified reagents for the Mitsuda test, or as a source for solubilized proteins for leprolins.