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Advances in Leprosy Research

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INTRODUCTION

In January, 1965, President Johnson and Prime Minister Sato of Japan agreed to undertake a greatly expanded programme of co-operation between the United States and Japan in the field of medical science. Their resolve was speedily implemented through the newly formed United States—Japan Co-operative Medical Science Programme. Primary emphasis of this Programme is on medical research on diseases of special concern to Asia. Leprosy is one of these. A joint panel of American and Japanese scientists found itself in complete agreement that the following areas in leprosy deserve special attention:—

1. Multiplication of the leprosy bacillus in:
   (*a*) artificial culture media;
   (*b*) cells isolated from man and other species;
   (*c*) the intact animal.
2. Drugs against the leprosy bacillus, treatment of reactive episodes during the course of the disease, and drugs suitable for chemoprophylaxis.
3. Vaccination as a control method.
4. Certain aspects of the immunology of leprosy, including the pathogenesis of the reactive episodes.

During the last few years there were several occasions where advances made in these particular areas of leprosy research were reviewed where road blocks impeding progress were identified, together with singposts leading to new and promising approaches. Among these occasions were:—

The Leonard Wood Memorial Foundation (LWM)-John Hopkins University Symposium on Research in Leprosy, Baltimore, 1961.

The VIII International Congress of Leprology in Rio de Janeiro, 1963.

The LWM-Armed Forces Institute of Pathology Conference on Research Problems in Leprosy, Washington, 1965.

The International Conference arranged by the Leprosy Panel of the U.S.-Japan Joint Medical Science Programme, Tokyo, 1966.

The major accomplishments brought to light, their extensions and applications, as well as emerging guiding concepts for future work, are the subject of this review.

This report, therefore, is selective and does not deal with all the aspects of leprosy. Excluded, for example, are the splendid efforts and advances made by Paul Brand and his co-workers in the use of physical and surgical methods in the prevention and management of deformities in leprosy. Also excluded is a detailed discussion of recent clinical drug trials.

It appears convenient to divide the subject into two major sections. Firstly, multiplication of *M. leprae* in artificial culture media, in cells isolated from man and other species and in the intact animal will be discussed.

This part of the discussion will point up some characteristics of the leprosy bacillus which now serve to identify it. It will illustrate on-going basic research, present-day application to experimental chemotherapy, active immunization, and epidemiology.

The second section concerns itself with host-responses to infection, such as resistance and hypersensitivity. This part of the presentation establishes a theoretical basis for a better understanding of the different clinical and corresponding histopathological types of leprosy, and of newly evolving concepts of the pathogenesis of the reactive episodes and the urgent need for supporting them with experimental evidence.

SECTION I
Attempts at Growth of *M. leprae* Outside of its Natural Host

At the Conference on Leprosy Research Problems in 1965 in Washington, D.C., the greatest amount of time was allotted to the cultivation problem of *M. leprae*. The keynote of the Conference seemed to be how incredible it was that in spite of all microbiological advances the leprosy bacillus stills fails to multiply *in vitro*. It is, of course, well known that successful cultivation of a microbial pathogen ordinarily precedes an increase in our understanding of the pathogenesis, epidemiology, treatment, and prevention of an infectious disease. It has been voiced repeatedly that progress in gaining a better understanding of leprosy has been hampered by the fact that *M. leprae* has not yet been grown in bacteriological culture media.

(*a*) *Attempts at Growth of M. leprae in Artificial Culture Media*

Solution to the problem of growth of *M. leprae* in bacteriological culture media is now being approached directly by making additives to basal media, in the hope of supplying thereby the needed growth-factors. One reads or hears occasionally reports of success (Alexander-Jackson, 1961; Olitzky and Gershon, 1965; Nakayama, 1966), but in not a single instance were the claims supported by up-to-date criteria

It would not seem too promising to invest much time and resources in producing culture media by incorporating into a given basal medium chemical compounds selected at random. Obviously, the choice of candidate substances is legion, and mathematical considerations point up the vast number of different media which are possible by combining substances from even relatively small numbers of choices. Attempts at culture should be supported by rational working concepts. Lately, there has been increasing evidence that future additives to basal media will rely largely on components and co-factors which now have been revealed as important in the structure and function of mycobacterial cells other than *M. leprae*. (Morrison, 1965; Wheeler and Hanks, 1965; Hanks, 1965.) It seems that more direct evidence for suitable additives might result from metabolic studies with *M. leprae*. Due to lack of adequate amounts of material, no biochemical studies have been conducted until recently on *M. leprae*, and its metabolism remained completely unknown. This, in turn, has led to empirical methods being applied to cultivation of the bacillus and in chemotherapy of the disease. Now, however, it has been shown that the leprosy bacillus actively oxidizes 3, 4 dihydroxyphenylalanine (dopa) and that this enzymatic activity clearly distinguishes *M. leprae* from other mycobacteria, including *Mycobacterium lepraemurium* and microorganisms which at one time were thought to be the leprosy bacillus. (Prabhakaran, 1967, a, b; Prabhakaran and Kirchheimer, 1966.) This activity of *M. leprae* has been proposed by Prabhakaran and Kirchheimer (1966) as one of its identifying characteristics. A detailed investigation of the possible function of quinones produced by dopa oxidation as respiratory carriers is now under way in our laboratories. If dopa oxidation proves to be a key reaction in the respiration of *M. leprae*, rational approaches to artificial culture and anti-microbial therapy become possible.

(*b*) *Attempts at Culture of M. leprae in Tissue Culture*

Successful cell culture of *M. leprae* can be looked upon as a long step forward toward eventual independent growth of this microorganism. This topic is receiving major attention in several laboratories. The most encouraging results so far were reported in 1965 by Garbutt. Using monolayer cultures of human embryonic long cells, she obtained a 148.6 fold increase in bacterial numbers in 141 days. This corresponds to 7.1 bacterial generations, with an overall generation time of 17.4 days. In an additional experiment with 14 p f rat fibroblasts she obtained a cumulative bacterial increase of 1.62×10^6 fold, or 20.5 generations over 452 days, giving a generation time of 22.0 days. The general principles and methods used in these experiments were very much like the ones

previously used by Garbutt, Rees and Barr (1962) and Rees and Garbutt (1962) in their successful attempts to grow *M. lepraemurium* in 14 p f rat fibroblasts. Methods used in our own experiments in Carville are similar to those used by the English workers. Human, other mammalian cells, and those derived from poikilothermic animals (rainbow trout) are maintained in such a form that they permit continuous cycles of cellular infection. An attempt is made to maintain the infected cells in media insuring multiplication at slow rates, to maintain favourable cell/bacteria ratios. In addition, substances are added to the cell culture medium, which might stimulate multiplication of the leprosy bacillus.

Among candidate agents which might be found useful for 'unblocking' *M. leprae* are: Diaminopimelic acid, mycobactin, RNA from various sources, gibberillic acid, -SH group donors like cystein, and more heterogeneous substances like sterile extracts of lepromatous tissue.

Evidence obtained from multiplication of *M. leprae* in the mouse foot-pad makes it appear likely that its growth temperature requirements might resemble those of *Mycobacterium balnei* and *Mycobacterium ulcerans*. On account of this possibility, incubation is at 33°C. and 37°C. Furthermore, it cannot be excluded that a relatively high pO_2 might be injurious to obligate intracellular parasites. This, of course, invites experimentation with gas phases of various composition, particularly some with increased p CO_2.

Up to this time, only small numerical increases were observed. For example, in cultures of human embryonic lung cells at 37°C., containing 10^{-4}mg per ml of RNA from *Mycobacterium phlei*, there was a 3.2 fold increase of the bacteria in 108 days. Small numerical increases of the acid-fast bacteria were also noted by Chang and Neikirk (1965). Their cell cultures consist of mouse peritoneal macrophages, supplemented with L fraction of liver extract (Nutritional Biochemical Corp., Cleveland, Ohio) and ferric nitrate, maintained in a 5% CO_2-air mixture.

(*c*) *Multiplication of M. leprae in Experimental Animals*

It is now well established that *M. leprae* multiplies in the foot-pads of mice (Shepard, 1960, 1960a; Janssens and Pattyn, 1963; Kirchheimer, 1964; Rees, 1964) and that maximum multiplication is limited to a narrow range of relatively low ambient temperatures (Shepard, 1965). The amount of multiplication and the location of the bacteria, following inoculation of several thousand leprosy bacilli into the foot-pad, is very typical and serves in distinguishing *M. leprae* from other mycobacteria, particularly *M. lepraemurium*. In contrast to the latter organism, *M. leprae* does not cause grossly visible changes of the injected foot, fails to invade adjacent or remote tissues, has a multiplication ceiling of a few million, and, as stated before, increased ambient temperatures depress its multiplication, which is not the case with *M. lepraemurium*. Recently, Rees (1965) has shown that in thymectomized, total body irradiated mice, kept alive with bone marrow transfusions, increased multiplication of the leprosy bacillus takes place in the foot-pad without, however, resulting in systemic infection. That the restricted multiplication of *M. leprae* in the foot-pad in all likelihood is due to immune intervention has been concluded from the observation that transfusion of thymectomized and irradiated mice with normal homologous lymphocytes re-establishes the former status, with respect to multiplication of the leprosy bacillus (Rees and Waters, 1966). Our own observation that multiplication of *M. leprae* in the foot-pad is depressed significantly in offsprings of vaccinated mice support an immunological explanation for the characteristic multiplication ceiling (Matsuo and Kirchheimer, 1966). Because of the quantitative and qualitative limitations of the mouse foot-pad model, search for more suitable experimental conditions is needed. In this connection it is noteworthy that Rees and Water (1965) noted a more generalized infection with *M. leprae* involving the foot-pads, ears, lymph nodes, striated muscles and tail skin in intravenous challenged thymectomized

and irradiated mice. In view of what was said before about the dopa oxidase activity of the leprosy bacillus, we are now contemplating trials with mice bearing melanomas.

Before summarizing some of the applications which were made of the mouse foot-pad model and some of the results of these endeavours, a third distinguishing characteristic of *M. leprae* must be added to its capability to oxidize dopa, and its multiplication pattern in mouse foot-pads. *M. leprae* seems to be the only known mycobacterium which fails to evoke grossly visible responses in the skin of leprosy patients with negative Mitsuda tests (McFadzean, 1962; Shepard and Guinto, 1963). Identification as *M. leprae* of an unknown mycobacterium failing to grow under ordinary conditions requires simultaneous intradermal injection of leprosy patients with the heat-killed mycobacteria and with standard lepromin. The leprosy patients must be so selected that there is a significant number of Mitsuda positive persons among them. In contrast to Mitsuda negative patients, these will respond with local reactions to the injection of leprosy bacilli.

(*d*) *Application of the Mouse Foot-pad Model*

The following is a list of applications which have been made of the mouse foot-pad technique:—

1. Experimental chemotherapy.
   Screening of anti-leprosy drugs.
   Detection of drug resistance.
2. Vaccination against *M. leprae*.
3. Stability of *M. leprae in vitro*.
   Effect of wet-ice temperature on subsequent multiplication.
   Effect of preservation at the temperature of liquid nitrogen on subsequent multiplication.
4. Epidemiology.
   Role of arthropods in the transmission of leprosy.

The fact that *M. leprae* multiplies with regularity in the mouse foot-pad has provided an experimental *in vivo* method for testing the drug sensitivity of strains of leprosy bacilli from untreated and treated patients. Drug sensitivity has been assessed by comparing the yield of bacilli in the foot-pads of treated and untreated mice. The respective drugs are either fed in the diet or given by injection, ordinarily commencing on the day of infection (Shepard and Chang, 1964; Shepard, 1964; Pettit and Rees, 1964; Rees, 1966; Shepard, 1966; Shepard, McRae and Habas, 1966).

The principal results of these tests show:—

1. That all the accepted anti-leprosy drugs, including diaminodiphenylsulfone (DDS), the thiourea (Ciba 1906), the thiosemicarbazone (TBI), the 2 long-acting sulphonamides, Fanasil and Madribon, and the phenazine derivative B 663, more or less completely inhibit the multiplication of *M. leprae* in the mouse foot-pad.
2. That *M. leprae* is extraordinarily sensitive to DDS (Shepard, McRae and Habas, 1966). As little as 0.0001% of DDS in the diet of mice completely suppresses multiplication of *M. leprae* in their foot-pads. From the available findings Shepard (1966) estimated that the minimum inhibitory concentration of DDS for *M. leprae* is approximately 0.03 gamma per ml of blood.
3. That leprosy bacilli resistant against DDS occur (Pettit and Rees, 1964).

Multiplication of *M. leprae* in the foot-pad of mice has also been exploited for determining the efficacy of anti-leprosy vaccines (Shepard, 1965, 1966). The criterion for protection is reduction of multiplication of leprosy bacilli in the foot-pad of vaccinated, as compared to non-vaccinated mice. It has been shown that intradermal injection of 50 mcg. (dry weight) of BCG, Rosenthal, given one to two months prior to challenge reduces the final crop of leprosy bacilli by 50%. Much smaller amounts of the vaccine still have measurable effects. Answers to the question of the prophylactic value of BCG vaccination for the prevention of leprosy are being sought in mass trials in Uganda, Burma and New Guinea. The Uganda trial (Brown and Stone, 1966) included about 16,000 tuberculin-negative children, mostly under 10 years old and all in contact with leprosy. Half

of the children not vaccinated served as controls. The other half received freeze-dried BCG. One to three years after vaccination 89 cases of leprosy were detected among controls, but only 18 among vaccinated children. Evaluation of these findings, however, must take into consideration that only 8% of leprosy in Uganda is of the lepromatous type. To be certain that BCG vaccination is likewise effective in reducing the incidence of lepromatous leprosy, one must await the results of the trials from areas like Burma, which has a high incidence of lepromatous leprosy. Results from these fields trials in Burma and New Guinea will not be known for 2 or 3 years.

Shepard and McRae (1965) used the mouse foot-pad system to determine the effect of low temperatures and freezing on the infectivity of *M. leprae*. The keeping-quality of the leprosy bacillus is of special importance to leprosy research. This becomes understandable if one remembers the fact that competent research laboratories frequently are located far from the sources of supply of suitable material, which consequently must be shipped long distances. As measured by their subsequent ability to multiply in mouse foot-pads, leprosy bacilli in balanced salt solution containing 0.1% bovine albumin maintain viability for about 2 weeks at a temperature of 0°C. Freezing and storage at –60°C. does not seem to be a good way to preserve the leprosy bacillus. It is possible, however, to reduce the deleterious effect by including 10% of glycerin in the suspending medium prior to freezing. Experiments on viability of leprosy bacilli stored at the temperature of liquid nitrogen (–193°C.) are now under way.

At the VIII International Congress of Leprology (Rio de Janeiro, 1963) the members of the Panel on Epidemiology and Control expressed their opinion that the control of leprosy is closely dependent on the present state of knowledge concerning the epidemiology of the disease. They believe that a better understanding of the mechanism of transmission may bring about basic changes in our present method of control.

The unsatisfactory state of knowledge of the mode of transmission of leprosy is pointed up by extant disagreements about the portal of entry of *M. leprae* into the human body. Recently, Weddell and his associates (1963) have cast doubt upon the belief in the prevalence of the dermal route of infection subscribed to by most leprologists. Furthermore, it is worth noting that not even the proponents of the hypothesis of dermal entrance are in agreement among themselves on the nature of the infecting event.

Some, like Khanolkar (1963), have stressed the necessity of persistent and intimate contact with human cases of leprosy in an infectious state. Dungal (1960,1961), on the other hand, comes out in favour of accidental infection by ectoparasites or parasites of the skin. One must agree with Spickett (1961) that a likely vector would have to be able to penetrate to the subepidermal tissues of man, the ordinary location of *M. leprae*, and in addition must be able to support the prolonged survival of the leprosy bacilli, particularly in its alimentary tract. Effective role as a vector also depends on biological characteristics of an arthropod such as feeding habits and length of survival, as well as on the behavioural characteristics of its host.

In the past, several workers (Muños Rivas, 1942; McCoy and Clegg, 1949; Spickett, 1961) have reported the occurrence of acid-fast bacilli in the alimentary tract of arthropods. Muños Rivas, for example, found 187 (11.4%) carriers of acid-fast bacteria among 1,627 fleas taken from places free of leprosy. At the time these reports were made, and in fact until recently, it was not possible to either identify these bacteria as *M. leprae* or prove their viability. In consequence, the significance of these findings for the transmission of leprosy could not be assessed. Fortunately, no such handicaps exist at the present time. Now, it is known that perhaps even as few as 10 viable leprosy bacilli, if injected into the mouse foot-pad, will multiply at this particular site, provided the ambient temperature of the animal quarters does not deviate much from 70°F (21°C). Additional methods for identifying

mycobacteria growing in the mouse foot-pad as the leprosy bacillus have been discussed before. A research project, entitled 'The Role of Arthropods in the Transmission of Leprosy,' is now getting under way, in collaboration between the United States Public Health Service and The Jawaharlal Institute for Postgraduate Medical Education and Research, Pondicherry, India.

SECTION II

Host Responses to Infection

It is a common observation that individuals exposed to leprosy bacilli may fail to develop readily detectable signs of infection. Others display partial resistance to the leprosy bacillus by supporting its growth in their tissues only with reluctance, or for a limited time. The tissues of some individuals, however, seem quite incapable of any effective resistance to the multiplication of the invaders and their progeny.

At the VIIIth International Congress of Leprology in Rio de Janeiro in 1963, there was an increasing readiness of workers in leprology to regard the various clinical and corresponding histological types of leprosy as an expression of the amount of host resistance. The 2 polar types of leprosy, tuberculoid and lepromatous, respectively, represent high and low degrees of resistance and stand at the ends of a spectrum encompassing all of the variations in resistance, expressing themselves as dimorphous (borderline) leprosy with varying amounts of mixtures of both forms.

The question would now arise as to the kind of mechanism to which different individuals owe their particular degree of reistance to the leprosy bacillus. At the Congress, the members of the Sub-Committee on Immunology of the Panel on Bacteriology and Immunology expressed the opinion that native and acquired resistance to mycobacterial infections have not been shown to be due to or associated with antibody or antibody production. They stated, furthermore, that native resistance may depend in part on factors unfavourable to the reproduction of *M. leprae*. Native resistance, in the opinion of the members of the sub-committee, becomes rapidly fortified by acquired resistance. The latter was said to consist primarily of an improvement in the natural capacities of mesenchymal cells to digest mycobacterial cell walls and to hydrolyze their proteinaceous components. They added that acquired resistance to mycobacterial disease seems analogous to induced formation of enzymes. It is, of course, reasonable to assume that the extent to which individuals might respond with adaptive enzyme formation is genetically determined. There is so far no experimental support for this hypothesis as far as leprosy is concerned. In experimental tuberculosis, however, it has been shown that mononuclear phagocytes of rabbits genetically resistant to tubercle bacilli had greater metabolic activity on certain substrates than the same cells of susceptible animals (Allison, *et al.*, 1963). In particular, cells from resistant animals showed a greater ability to break down glycerophosphate and Beta-hydroxybutyrate. Both of these substances are linked to the scheme of lipid metabolism. Lipids, of course, are an integral and predominant part of tubercle bacilli and some of these like, for example, the 'cord factor' may play a role in virulence. This increased metabolic activity of mesenchymal cells might, therefore, be of importance for explaining increased resistance against tubercle bacilli. Note must, however, be taken of the recently demonstrated fact that antibodies against antigens of the leprosy bacillus, identified as immunoglobulins, are present in the serum of patients with lepromatous and tuberculoid forms of leprosy (Merklen, *et al.*, 1963). There are indeed indications that the resistance or the susceptibility to the disease may be related to immune phenomena. As was pointed out earlier, in the mouse foot-pad, at least multiplication of *M. leprae* seems to be limited because classic immune mechanisms are being invoked. Furthermore, there are several reports of abnormalities in the serum proteins in leprosy, particularly abnormalities of the immunoglobulins (Nudenberg, 1965). Matthews and Trautman (1965) demonstrated the presence of cryoglobulin in the sera of all 39 individuals with lepromatous

leprosy who were not receiving corticosteroids and in 16 out of 29 who were at that time under treatment with corticosteroids. This finding, associated with an increase of globulins in the gamma zone and the seeming lack of proper defence mechanisms, becomes extremely important in view of the usual association of cryoglobulins with immunologically incompetent paraproteins (Grabar and Burtin, 1964). Evidently, the antibodies found so far are not connected with resistance to the disease, since they prevail in people who do not exhibit a satisfactory immunological response. In the absence of a demonstration of protective antibodies, a study of the structure of the antibody-active fragment of the immunoglobulins is the only possible approach to a study of immunological differences between apparently resistant and susceptible subjects.

An important and in many respects enigmatic aspect of leprosy is the pathogenesis of the reactive episodes. The need for clarification of the mechanism of these reactions was clearly recognized and expressed by the Panel on Leprosy Reactions of the VIIIth International Congress of Leprology (Rio de Janeiro, 1963). In the opinion of the majority of leprologists these reactions are allergic manifestations to mycobacterial antigens. The significance of mycobacterial antigens as possible sensitizing agents can, of course, not be denied. Yet it would seem best not to dismiss the possibility that, in some instances at least, other antigens might have engendered the hypersensitive state and, therefore, are capable of eliciting reactions at a later date. Some theoretical considerations support the assumption that autosensitization may take part in the pathogenesis of lepra reactions. For instance, the enhancing action of mycobacterial bodies on the antigenicity of other substances is well known and has been widely exploited experimentally in the form of Freund's adjuvant. One can well imagine that in leprosy mycobacterial bodies may exert a similar effect on tissue components which might have undergone some alterations during the prolonged course of the disease. In addition to support derived from theoretical considerations, the following observations have been interpreted as suggestive of 'auto-immune' involvement: false positive serological reactions with lipoidal antigens, occurrence of antithyroid antibody, rheumatoid factor, antinuclear antibodies, and cryoglobulins (Cochrane, 1964;, Matthews and Trautman, 1965).

SUMMARY AND CONCLUSIONS

1. Research in the areas of cultivation of *M. leprae* chemotherapy, chemoprophylaxis, vaccination and of the immunology of leprosy is being accelerated under the auspices of the U.S.-Japan Co-operative Medical Science Programme.

2. The renewed attempts at cultivation in artificial culture media are guided by newly evolved concepts. Attempts to culture the leprosy bacillus in cell culture have provided some encouraging results.

3. The typical multiplication pattern in the mouse foot-pad, the distinctive reaction pattern of Mitsuda positive and negative individuals to intradermal injection of leprosy bacilli and its unique enzymatic activity distinguish *M. leprae* from other mycobacteria.

4. The mouse foot-pad system is being employed for the study of various aspects of leprosy like drug action, efficacy of vaccines, environmental effects on bacterial survival, and certain epidemiological aspects.

5. Modern immunological techniques need to be employed to further the understanding of resistance and the role of hypersensitivity.

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