BACTERIOLOGY AND PATHOLOGY

13 NOVEMBER, 1958
9.15–12.00

Chairman: DR. J. H. HANKS

Rapporteur: DR. R. J. W. REES

DR. HANKS described the preliminary work of the Panel and Technical Committee, and reported that in *Bacteriology, immunology and pathology* there were limited contributions; but there has been progress in many ways:—

Bacteriology

1. Methods for counting bacilli have been developed and are of great value. Techniques were described by Rees, Hanks and others.
2. Cultures of bacilli have recently been partially successful. Other mycobacteria, e.g., of tuberculosis also have difficulties in cultures. Tissue cultures are more successful in *M. leprae*. Dr. Rees will discuss this. Two problems in culture: to find tissue cells of other host systems to substitute for natural ones: or to modify the nutritional or hormonal balances. Assessment of results is difficult. Enumeration of bacilli and histological assessment can be used. Microbiology and genetics and electron-microscopy are proving helpful. These must have other mycobacteria as comparison.

Pathology

Superficial nerves and skin and endothelial system in leprosy are prominent. There is an effect of bacteriaemia. Pathology is also important in prognosis and therapy. Great progress has been made in histology, using Fite-Faraco process for demonstration of bacilli in sections.

Diagnosis consists of demonstrating

(*a*) acidfast bacilli or host cell

(*b*) cellular invasion or around the nerve fibril

(*c*) histological studies for classification and prognosis

i Tuberculoid—focal infiltration of epithelioid or lymphocytic cells.

ii Lepromatous—granulomatous infiltration of lymphocytic cells not reaching into subepidermal zone.

iii Intermediate types—a mixed picture but requires more clarification.

Histology in treatment is valuable to study behaviour of cells and bacilli—an Index can be compiled (Ridley). This should be taken up by pathologists and agreement reached.

Early histology of nerves may be studied to find out if this is earliest region of invasion or not.

Lepra Reaction Erythema nodosum type, and reactional type: they are not bacillary in origin, but reaction of the body. Further study much needed.

DR. R. J. W. REES: (U.K.)

The Study of Rat Leprosy in Tissue Culture

Previous attempts at culture having failed, studies of other strains should be useful. Rat leprosy is chosen because in many ways it is similar, and *M. leprae murium* has not previously been cultured and would provide a model system. A tissue culture system is a hopeful system for growing it for many reasons. Serum concentrations should be reduced, and generation time discovered. The latter has been found to be 10–12 days (*M. tuberculosis* 24–30 hrs. *in vivo*). Bacilli may persist alive or dead and lead to error. Methods of counting total numbers of bacilli before or after culture have been developed by us.

Monocyte culture after 80 days: no multiplication but viable bacilli seen.

Spleen explants offered a hopeful method of culture and limited multiplication was shown. Addition of streptomycin to INH inhibited the multiplication.

Similar results from separated spleen cells.

Fibroblast cultures have given best results so far. The bacilli do multiply, as shown by enumerations, up to 60 days. This may be a basis for advance.

DR. HANKS said his own work coincides with that of Rees. An important point is not to count the cells but to count the bacilli. A disappointing feature is that the multiplication is so limited: an essential component still seems to be lacking and we do not think vaccines from cell cultures will ever be possible. But cell culture is a tool for analysis of many problems.

DR. J. H. HANKS (U.S.A.):

Evaluation of Physiological State of M. Leprae by Cytology

Methods of evaluation of *M. leprae* and its physiology have been developed. We first used tetrazolium compounds. The amount of pigment produced indicated metabolic activity, and could be used to say if bacteria were alive or dead. Tetrazolium was used to stain bacteria, and blue granules meant there was metabolic activity. With pathogenic species the proportion did not agree with *pathogenic activity.*

Saffranin was added to tetrazolium: *permeation by dye means no metabolic activity*, but 5% remain a mystery. Other enzymes and solvents were tried but would not modify permeability except in presence of heat. The impermeable bacteria were the active alive ones. Pathogenic bacteria do not admit the tetrazolium agent which therefore will not be useful to us.

Dyes have been studied: pathogens have a wider range of permeabilities: some produce DNA granules, especially *crystal violet*. *M. leprae* have non-permeable types to a dye at 37°C. There are great variations in permeability of mycobacteria: the saprophytic type are permeated readily, *M. leprae* very slowly. Capsulation seems an essential factor: electronmicroscope shows a definite envelope.

Use of dyes in presence of heat is therefore a useful test of metabolic activity of mycobacteria. The malachite green method illustrates this: it attacks DNA.

Discussion

DR. CHATTERJEE said: "The impermeability of the cell wall of leprosy bacilli, described by Dr. Hanks, may possibly be due to the presence of complex calcium bound carboxylic groups not only in the cell wall but also in the gloea around each bacillus. This cell wall contains oxy-fatty acid insoluble even in petroleum ether.

"However, by hydrolysis with 0.1 N HCl for about 12–15 minutes at 56°C or with N HCl for 10 minutes at 37° C, often many bacilli are rendered permeable due, probably, to the removal of some of the loosely bound calcium from the cell wall and gloea."

DR. HANKS replied. He said he found he could not stain the capsules and was glad to hear Dr. Chatterjee's contribution.

DR. D. H. BINFORD (U.S.A.):

Histiocytic granulomatous mycobacterial lesions in golden hamsters inoculated with M. leprae: negative results in 10 experiments in other animals.

He reported inoculation experiments in animals, and described materials and methods. He thinks that *M. leprae* selectively affects the cooler parts of the human body. On inoculation of golden hamsters the characteristic picture was a *histiocytic granuloma* (in histological section of testis). There was always a pattern of increased histiocytes. Some contained bacilli. One animal had a nodule on one ear: it had numerous intracellular bacilli.

Intratesticular and intra-auricular inoculations showed similar results, the histocytic granuloma.

Small nerves also showed numerous bacilli. The lesions were of slow progress. No foam cells nor globi were seen but the bacilli intracellularly were numerous. Nerves were invaded. The work will continue.

DR. K. R. CHATTERJEE (India):

Experimental transmission of human leprosy to a hybrid strain of black mice

He first acknowledged his debt to Dr. Hanks, Dr. Fite, and other workers. The root of the problem was to find a new experimental animal and to introduce some human factor along with the inoculation of the bacilli.

The animal was the laboratory crossbred black mouse, very young, 10–15 days old. He chose the black because of fancy only. After thirteenth generation only black come. He has used 106 such

animals: 6 lost, so 100 left. More than 50% took up infection by 14 months, a heavy infection.

He separated the bacilli by differential centrifugalization and counted them and gave only 1 inoculation of 20 million bacilli subcutaneously in various regions of the animal. After 6 months he found multiplication in the animals. Animals which died were examined. There were dryness of skin, loss of hair, some nodules, spleen and liver were enlarged, inguinal glands enlarged. Microscopically fuchsinophil cells in tissues were early to be found, before bacilli were seen. Later bacilli became numerous inside liver cells, and round blood vessels, in spleen (great number in spleen), cells and globi numerous everywhere, numerous in lymph glands and testis, nerves, etc. Could it be a saprophyte? Various media were used for culture—all negative. Lepromin by Dharmendra technique was tested against the inoculated bacillary lepromin and found parallel in reactions.

Inoculation of *M. leprae* to these mice thus seems to be successful.

Discussion

DR. BINFORD commented how he urged Dr. Chatterjee to come to the Congress because of importance of having a laboratory animal to which leprosy can be transmitted. He asks if the black mouse could be made available in other parts of the world.

DR. WADE. "From the reports by Drs. Binford and Chatterjee that we have heard, and of other reports by Dr. Bergel of Argentina which have been published (one of them now in press in the International Journal), it would seem that at long last there has been a break-through in the problem of producing bacillus-loaded granulomatous lesions in animals with inocula from leprosy lesions. Animal granulomas such as have been shown could not have been produced with heat-killed leprosy bacilli. Chatterjee and Bergel have reported inability to obtain cultures from their experimental lesions, and also lepromin test with suspensions of such lesions with results (negative in lepromatous cases and positive in tuberculoid cases) such as are obtained with human lepromin. These results are highly encouraging and if confirmed will mark a new phase of the experimental study of leprosy.

DR. REES commented: "I fully support Dr. Binford that every attempt should be made by instructed groups to repeat these successful transmission experiments reported this morning. With this object in mind I should say that I have, in the past four years, been occupied in attempts to transmit human leproma to mice, with completely negative results. I have used albino mice and like Dr. Chatterjee attempted to separate bacilli from the lepromatous tissue. My negative result may therefore suggest either (1) my unsuitable mouse strain, or (2) unsatisfactory separation of tissue. Could Dr. Chatterjee therefore say how important the mouse strain may be? Has he tried any other strains of mice and with what result?"

DR. CHATTERJEE replied: "I have used white, and mice of other colours but without positive results. It is the black strain only. I hope it will be possible to supply the animals to other workers interested in this type of experiment. A formal request may kindly be made to the Director, School of Tropical Medicine, Calcutta–12, India."

In reply to Dr. Rees he said: "We have often tried other animals also. Of these, laboratory-bred Syrian hamsters have been found to take up infection to some extent. The lesions generally remain localized for quite a long time after which generalised dissemination of a mild type has been observed in a small number of hamsters; only few of them showed moderately heavy infections when the animals survived a long period after inoculation. So far the number of hamsters which take such infection is so small that it does not justify any definite conclusions. It may therefore be said that till now only the black mice have been found to be susceptible to infection with human leprosy."

DR. AZULAY, Brazil asked about control groups and Dr. Chatterjee replied: "Control groups have shown negative results. Our inoculum contained 1 to 20 million bacilli only and the animals sacrificed after about 8 months and later showed the tissues crammed with innumerable bacilli, which indicated definite multiplication of the organism in them."

DR. BINFORD replied to a question on controls in his work: "Controls of heat killed inoculum were given: such showed nothing but the carbon ink in the control inoculum: no granulomatous process was seen."

DR. D. S. RIDLEY (U.K.):

Observations of prognosis of different classes of leprosy in different races

Difficulty arises in clinical estimation of progress (under treatment) of different races. There are errors even in using the bacillary index because of where one reaches in the skin with taking material for a smear, so one may miss the improvement or bias to a reading of lack of improvement.

But *biopsy* is more useful. The granulomatous part of the lesion can be identified and the bacilli counted as well, and arithmetical factors given for each of the two important criteria of progress (bacillary count and histological picture).

In different regions of the world lepromata differ and they may even differ in parts of those regions. Bacterial index also varies in typical cases. But the new serial biopsy gives fall at standard rate, *i.e.*, 25% in Europeans in 6 months; Anglo-Indian rate is the same. The other races—not enough figures are obtained yet. This all applies to leproma and there is remarkable uniformity. Histologically the estimation of the figure for the picture is quite easy and practicable. Borderline and intermediate cases are harder to estimate. Lymphocytic reaction is less in E. Africa than in W. Africa. Fibrosis in a lesion represents rapid resolution. Lymphocytic infiltration is often followed by fall in bacterial density. Borderline respond to treatment twice as fast as lepromatous: It is a matter of individual resistance.

Discussion

DR. HANKS: "Dr. Ridley's paper gives a new view, and the use of his serial biopsy index should be studied widely."

DR. WADE: "During the last several years at Culion I have been observing a nonfoamy-cell variety of leproma (which I call 'histioid' because it resembles superficially an organized tissue rather than a granuloma) which might be well represented by the slide which Dr. Ridley described so heavily infiltrated with fibrocytes. It was impossible, as hoped, to prepare a formal paper on the subject for presentation, but through the courtesy of Professor Kitamura several photomicrographs are on exhibit in the demonstration room, to which the attention of interested members is invited. The most interesting features are the spindle shape of the histiocytes, the general resemblance of the chronic lesions to fibrous or fibromatous lesions, and above all by the fact that although the histiocytes are crowded with bacilli there is—typically—no production of gloeal substance and consequently no globi or foamy cells."

DR. MONTESTRUC: "In Martinique the lepromatous cases do show a fibromatous element in histology, in cases which respond very well to sulphone treatment."

DR. HANKS: "Afternoon session will be at 1 p.m."

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1.20 P.M. TO 3.30 P.M.

DR. C. C. SHEPARD (U.S.A.):

Tissue Culture of Mycobacterial Pathogens

Various tissue cultures and techniques are described. Serum content (horse serum 10 or 11%) is used to make the virus techniques suitable for bacteria, on the monolayer system. Intracellular growth rates bear a direct relation to the growth rate of the species in culture. Work on *M. leprae* was inconclusive.

DR. R. F. NAYLOR (Uganda, East Africa):

Study of Action of Sulphones on the Metabolism of Mycobacteria

He conducted studies on *M. phlei* and *M. ranae* of DDS by tissue respiration. The results show only a modest depression of oxidation of substrates and little effect on growth. There is a bacteriostatic effect but no bactericidal effect. Production of PAB by the bacteria antagonizes action of sulphones. There is no evidence of sulphone-resistant mutants.

Resistant forms do not readily arise.

Dehydrogenase activity as index of viability as shown by Hanks was tried in some experiments with *M. leprae:* it was found to be present and to persist a long time under treatment.

DR. J. R. MCFADZEAN AND DR. R. C. VALENTINE (Malaya):

An attempt to determine the morphology of living and dead mycobacteria by electron microscopy

Studies on *M. leprae murium* and *M. leprae* by the electron microscope showed electron-dense bodies with an almost uniform electron density. These were untreated. The treated forms showed shrunken cytoplasm and empty cell membranes. Thus viable forms can be distinguished.

DRS. R. J. W. REES, R. C. VALENTINE AND P. C. WONG (U.K.):

Electronmicroscopic appearances of mycobacteria

They studied mycobacteria to confirm McFadzean's observations (in previous paper). For tubercle bacilli the same appearances were found and correlated with viability. There are 3 types, normal, degenerate, and segmented. The difference is based on the internal structure as seen by the electron microscope. The same was found with *M. leprae murium* and with *M. leprae* obtained from lepromatous tissue. The segmented form is referred to by some as spores, but no evidence of life was found in their experiments. They gave INH as therapy and found 73% degenerate (13% in untreated): later the degenerates rose to 95%. They use this in tissue culture systems to detect living and dead bacteria.

DR. S. OKADA (Japan):

Murine leprosy bacilli in electron-microscopic studies

He studied untreated bacilli, stained bacilli, those treated with chemicals or ferments, also ultra-thin sections of murine leprosy cells and bacilli.

He found that *M. leprae murium* has cell wall which is 10–20 mμ in thickness and a cell membrane, at least in part. There are also some granules which are electron-dense and may be mitochondria. The density increases at a zone of apparent cell-division. In the cytoplasm there are also microgranules 14 to 20 mμ in diameter, which can be isolated by centrifuge. These granules contain DNA and protein and are gram-positive. He thinks these microgranules are minimal structural units, and have chemical activity and produce the gram-positivity.

DR. S. W. A. KUPER (U.K.):

Skin reactions to lepromin and tuberculin: effect of BCG on histology

Tuberculin and lepromin tests in large series were done. In 105 healthy and 52 with lepromatous, etc., no correlation was found, but in tuberculosis a distinct correlation between the 2 tests was found In histology of lepromin injections in 150 patients there is only histiocyte proliferation in the lepromatous, but tuberculoid cases show a characteristic lymphocytic infiltration, often along with giant cells and tubercles. One could classify the disease quite well from the histological picture of the lepromin reaction.

BCG was given to see if it altered the reactions to lepromin. There was nothing much in normal subjects and tuberculoid cases, but in lepromatous leprosy 22 out of 30 patients showed an altered response, in the shape of a distinct trend towards the lymphocytic type of reaction, which suggests that a systemic immunological response had been elicited. The sensitivity to lepromin is a lymphocytic phenomenon.

Dr. Kuper and Dr. J. Robert May demonstrated their technique for the detection of Acid-Fast Bacilli by fluorescence microscopy. The equipment was set up in a demonstration room of the Congress and Dr. Kuper showed the results and described the technique. They use auramine-rhodamine. The bacilli appear brilliant orange in colour.

DR. Y. HAYASHI (Japan):

Phagocytosis of leprosy bacilli by leucocytes of leprosy patients, and phagocytosis of other acidfast bacilli

This study was to find out if leucocytes from leprosy patients have specific phagocytic power on leprosy bacilli. The results were obtained on 141 patients of all types of the disease and 20 healthy subjects. *The leucocytes from lepromatous* patients were found to be the strongest in phagocytosis (70% of an average), and least in polyneuritic (neural) patients, in whom it was 42.8%, and intermediate in tuberculoid patients (60.5%). In normal subjects the phagocytic activity was much weaker than in leprosy patients.

In an experiment with non-pathogenic acidfast bacilli, the lepromatous patients again were found to have the highest phagocytic activity, with tuberculoid and 'neural' least.

These findings can be useful to help in diagnosis, and are of interest in immunology.

DRS. T. YAMAMOTO, N. NISHIURA, N. HARADA AND T. IMAEDA: (Japan)

Electronmicroscopy of lepromatous and tuberculoid lesions

Ultrathin sections of lepromatous and tuberculoid lesions were made from skin and peripheral nerve trunks. In the lepra cells there were opaque droplets and foamy spaces between them; this leads to the 'foamy cell'. In tuberculoid lesions, in early stages of the reactional lesions, there are a few small opaque droplets in the phagocytes loaded with leprosy bacilli. The bacilli look identical in both tuberculoid and lepromatous leprosy. In tuberculoid leprosy the bacterial cell membrane is broken down, and mitochondria and microsomes escape from broken cells into the extracellular body fluid. In peripheral nerve trunks the Schwann cells seem to be active and bacilli lie in the endoneural spaces. There is Wallerian degeneration, tubercle formation, and bionecrosis in the nerve trunk, and necrosis. The electron transparent zones around *M. leprae* are a kind of biostabilizer for the bacilli.

DR. TOMOSABURO OGATA (Japan):

The Disease Types of Leprosy Studied from the basis of experimental pathology

Lepromatous lesions show a reaction similar to that of a foreign body; the tissue action is similar to that from a small foreign body. When the bacillary toxin escapes, we get the tuberculoid picture. The body resistance is always weak but stronger in tuberculoid and there are intermediary cases.

DR. P. BRAND (India):

Association between damage from leprosy and temperature

Dr. Binford's observations in pathology correspond with Dr. Brand's findings in surgical pathology. The author took many biopsies and studied tissues. Every tissue is affected by leprosy, except perhaps muscle. First comes infiltration, then granuloma. This process does not occur in infected tissues which are deep and away from the skin, even by a few cm. The maintenance of constant body temperature also seems to protect against infiltration and granuloma. Parts of body subject to cooling are those most subject to damage. On a tendon, macroscopically the damage looked like a fungus growth and would not be on the deep surface of the tendon but on the upper surface of it. Also where tendons cross, the deepening from the surface protects from infiltration. Some nerves never get paralysed, those that are deep, away from the surface of the skin, but the true criterion is that of infiltration. This occurs according to vertical distance from surface of skin. Another factor is ischaemia following infiltration of bundles of fibres: so thickness comes into it: a thick superficial nerve is more liable to be paralysed.

DR. JIRO MINATO (Japan):

Peripheral nerve involvement in upper limbs of leprosy patient (clinical)

The distal peripheral nerves are most often affected in sensory changes and most often the median nerve. Most motor paralysis is found below elbow and wrist. An order of frequency is given for thumb and hand paralyses and of individual flexors and extensors.

DR. GAY PRIETO AND DR. F. CONTRERAS (Spain):

Classification of Disability

They were surprised by lack of data of percentage of patients with deformities, so they attempted a classification on patients in Spain. This sort of information will be useful in evaluating the campaign. They had 3,600 patients under this sort of review and classified them according to disability. In 1,877 patients they found 40.8% in hospital. There was 53% disabilities in the hospitalized, this much less in those not hospitalized. In French West Africa there is a classification, such as perforating ulcer, loss of vision, etc. In our cases, mutilations were 25% in men, 15% in women (in the first grade of our classification). The significance of mutilation is obvious. The cost of dealing with them would be great everywhere. Even after eradication of the disease, there would be plenty of mutilations to be dealt with. We should think of this task.

DR. J. CHARDOME AND DR. M. LECHAT (Belgian Congo):

Arteriography of the feet in mutilated cases of leprosy

They found in mutilated cases of leprosy states of arteritis, arteriovenous communication, or arteriolar spasm. The drugs hydergine and priscol were effective. The conditions occur especially after prolonged sulphone therapy. It seems mutilation goes on in spite of sulphone treatment. The earliest sign seems to be decrease in blood flow, as shown by filiform appearance of the arteries.

DR. T. V. BANG AND DR. N. D. TEIP (Veitnam):

Arterial angiography in perforating ulcer

They have done 45 arteriographies in perforating ulcer. They found 43 with normal clinical pictures. For the artery in the abnormal cases, there was incomplete obstruction; or constriction of the calibre of the artery or distension of same; or enlargement of the collateral artery.

DR. S. LAI (Taiwan):

Experimental transmission of human leprosy to monkey by frequent long-term implantation

His first report on this work was in *Int. J. of Leprosy*, Vol. 23, No. 1, 1955. He now reports on lepromin reaction, bacillary examinations, sensation tests, and X-ray of the bones. This is all quite similar to human leprosy.

DR. J. H. HANKS (U.S.A.):

Enumeration of M. leprae for the standardization of lepromin

The author has devised an easy means of enumerating bacilli in lepromin. The bacilli must first be declumped and dispersed without protein precipitation or destruction of bacilli. This can be done by flowing 0.02 ml of chloroform beneath 0.2 ml of aliquots of well shaken lepromin: shake vigorously for 3 minutes. Then add 1% serum in water and use a standard platinum loop to transfer to clean slides in small droplets in rows. They are dried, formalized-stained, counted and assayed.

The Chairman, Dr. Binford, suggested discussion can now begin in the final few minutes, or comments be made on previous papers.

DR. ROLLIER (Morocco) congratulated the authors of the papers on arteriography and thinks they will lead to significant discoveries, and to linking up with other arterial diseases and to finding a treatment for perforating ulcer.

DR. K. R. CHATTERJEE(India) commented on Dr. Naylor's paper. Chatterjee used Kedrowsky bacillus and found the same result of bacteriostasis only. Also osmium fixation for electronmicroscopy he suggests. He suggests Sudan 3 technique in demonstration of lipids.

Dr. Chatterjee continued: "As far as the excellent paper by Drs. Prieto and Contreras is concerned, I would like to point out that in India proper classification with gradation of deformities in leprosy has been in practice for a long time. Epidemiological studies have shown that such types constitute about 5 to 10% of the total number of leprosy cases. In presulphone days occurrence of such deformed cases was more common especially in leprosaria, and progress of these could not be checked satisfactorily. But nowadays cases receive treatment before the development of deformity."

DR. REES (U.K.): "Dr. Chatterjee suggested osmium was a better fixative than formalin for looking at bacteria in the electronmicroscope. Our experience and we have compared them both, is that they are equally good for fixing whole bacilli. In fact for this work no fixative is really required for looking at freshly recovered organisms. We use formalin in order not to contaminate the apparatus with a potentially virulent organism. On the other hand addition of formalin is essential if leprosy bacilli cannot be worked at soon after recovery from the tissues in order to 'fix' the state of the bacilli. Without formalin the bacilli will transform to the degenerate form."

DR. BRAND (India): "It is important to distinguish arterial changes that may lead to ulceration, from arterial changes that result from ulceration and consequent scarring. In most ulcerated feet there is already a loss of sympathetic nerve and therefore a vasodilation. The loss of blood supply is likely to be due to local mechanical factors such as scarring and chronic oedema."

DR. BINFORD closed the meeting at 4.30 p.m. and thanked members for papers and cooperation in timing of papers.