Lepr. Rev. (1977) **48,** 269–281

Report of the Third IMMLEP Scientific Working Group Meeting

The Third Meeting of the IMMLEP Scientific Working Group took place on 21 to 25 February 1977. As in the case of previous Reports we are happy to reprint the Report of this Meeting, minus the Protocols, through the courtesy of the World Health Organisation.

List of Participants

Dr G. BARANTON, Institut Pasteur de la Guyane française, *97305 Cayenne Cedex,* Guyane française.
Dr B. R. BLOOM, Albert Einstein College of Medicine, *Bronx,* New York 10461, United States of America (Chairman).
Dr O. CLOSS, Institute for Experimental Medical Research, University of Oslo, Ulleval Hospital, *Oslo 1,* Norway.
Dr K. DAWIDOWICZ, Instituto Nacional de Dermatologia, *Caracas 101,* Venezuela.
Dr P. DRAPER, National Institute for Medical Research, Mill Hill, *London NW7 1AA,* United Kingdom.
Dr T. GODAL, The Radium Hospital, Montebello, *Oslo 3,* Norway.
Dr A. A. JUŠČENKO, Leprosy Research Institute, *Astrakhan,* U.S.S.R.
Dr W. F. KIRCHHEIMER, Laboratory Research Branch, United States Public Health Service Hospital, *Carville,* Louisiana 70721, United States of America.
Dr P. H. LAGRANGE, Institut Pasteur, 25 rue du Dr Roux, *75724 Paris Cedex 15,* France.
Professor E. LEDERER, Centre National de la Recherche scientifique, Institut de Chimie et des Substances naturelles, *91190 Gif-sur-Yvette,* France.
Dr M. J. LEFFORD, Trudeau Institute Inc., P.O. Box 59, *Saranac Lake,* New York 12983, United States of America.
Dr T. NAKAYAMA, National Institute for Leprosy Research, 1455, 4-chome, Aobacho, Higashi-Murayamashi, *Tokyo,* Japan.
Mrs M. E. PINARDI, Instituto Nacional de Dermatologia, Apartado Postal 4043, *Caracas 101,* Venezuela.
Dr R. J. W. REES. National Institute for Medical Research, Mill Hill, *London NW7 1AA,* United Kingdom (Rapporteur).
Dr G. A. W. ROOK, School of Pathology, Middlesex Hospital Medical School, Riding House Street, *London W1P 7LD,* United Kingdom.
Dr C. C. SHEPARD, Leprosy and Rickettsia Branch, Virology Division, Center for Disease Control, *Atlanta,* Georgia 30333, United States of America.
Dr J. L. STANFORD, School of Pathology, Middlesex Hospital Medical School, Riding House Street, *London W1P 7LD,* United Kingdom.
Dr G. STONER, Armauer Hansen Research Institute, P.O. Box 1005, *Addis Ababa,* Ethiopia.
Professor G. P. TALWAR, Department of Biochemistry, All India Institute of Medical Sciences, *New Delhi 110016,* India.

SECRETARIAT

Dr H. SANSARRICQ, Chief, Leprosy, WHO, Geneva.
Dr J. WALTER, Medical Officer, Leprosy, WHO, Geneva.
Dr G. TORRIGIANI, Chief, Immunology, WHO, Geneva.
Dr P. H. LAMBERT, Immunology, WHO, Geneva.
Dr K. L. HITZE, Chief, Tuberculosis and Respiratory Infections, WHO, Geneva.
Dr D. S. ROWE, Special Programme for Research and Training in Tropical Diseases, WHO, Geneva.

Summary Abstract

The Third IMMLEP Scientific Working Group (SWG) meeting, held in Geneva, from 21 to 25 February 1977, focused mainly on research projects in the immunoprophylaxis area of the scientific plan. Achievements since the second SWG meeting of December 1975, were reported and included:

(a) increased supply of *M. leprae* to the programme;
(b) further improvement of the method for increasing recovery and purification of bacilli from *M. leprae* infected armadillo tissues (Protocol 3/77);
(c) biochemical chracterization of cell walls from *M. leprae* and characterization of some of its antigens;
(d) identification of mycobacterial strains showing closer antigenic similarities to *M. leprae* than BCG;
(e) induction of cell-mediated immune reactions by killed *M. leprae* in guinea-pigs and mice;
(f) induction of resistance to *M. leprae* infection in mice with killed *M. leprae*;
(g) confirmation of earlier studies showing relatively low reactivity to *M. leprae* antigens in areas non-endemic for leprosy.

Problems which have arisen include the small numbers of *M. leprae* antigens from extracts of armadillo-grown *M. leprae,* the lack of absolute specificity of skin tests for mycobacterial infections and the poor immunogenicity of *M. leprae* related cultivable mycobacteria in some experimental animals. On the basis of these findings new Protocols included:

(a) methods for increased antigenic recovery of *M. leprae* from armadillos (Protocol 4/77);
(b) procedures for enhancing induction of cell-mediated immune reactions to *M. leprae* antigens (Protocol 5/77);
(c) procedures for enhancing induction of CMI and resistance to *M. leprae* related organisms by killed organisms (Protocol 7/77);
(d) procedures for enhancing resistance to *M. leprae* infections in mice (Protocols 5/77 and 6/77);
(e) re-examination of the capability of lepromins to sensitize subjects to *M. leprae* antigens by new methodology (Protocol 9/77);
(f) further improvements of skin-test reagents (Protocol 8/77).

The scientific progress made over the past year strengthens our initial hopes that a vaccine effective against leprosy can be developed; yet it must be recognized that fulfilment of that goal for practical, large-scale applications cannot be expected in the immediate future.

In reviewing the IMMLEP programme as a SWG pilot activity, possible ways by which SWGs may contribute to strengthening of research capability in leprosy endemic countries were discussed. The meeting recommended that:

(a) wherever possible, research fellows in the IMMLEP programme should come from leprosy endemic countries;
(b) there should be increased local training of personnel for field activities which could be important to future epidemiological or vaccine studies;
(c) interlinkage should be made between SWG meetings and training courses in leprosy endemic countries;
(d) WHO regional offices assist in recruiting research fellows and in arrangement of SWG meetings outside Geneva.

Summary Report

(1) INTRODUCTION

At the direction of the Steering Committee, the Third Scientific Working Group (SWG, previously designated Task Force) of IMMLEP met from 21 to 25 February 1977, in Geneva, to consider the results and achievements to date, but to focus particularly on the research projects related to the immunoprophylaxis aspects of the scientific programme. The SWG comprised 19 members in addition to 3 members of the WHO secretariat. With the exception of 3 Steering Committee members, only 4 had attended previous meetings, leaving 12 new members. Thus IMMLEP has again maintained its practice of regularly bringing in new members and selecting them according to the major aspect of the programme under consideration.

General report

Dr Lucas, Director of the Special Programme, stressed the importance and inevitability of the scientific research contributions coming, at least initially, from the developed countries, while underlining the importance of the training and institutional strengthening aspects of the programme for the developing countries in which the 6 selected diseases were major problems.

There followed a general discussion and assessment of the IMMLEP programme, its progress, fulfilment of targets, and balance between research and training as envisaged by TDR. Dr Godal (Chairman, IMMLEP Steering Committee) summarized as follows.

(a) Overall, the planned network programme was on target and progress had been very encouraging.
(b) However, there had been delays in progress due to:
    (i) changes in scientific staff at centres funded by IMMLEP;
    (ii) the discovery that some proteins of armadillos cross-reacted with those from man, a problem now overcome;
    (iii) delay in, and difficulty of, recruiting suitable nationals from countries where leprosy is endemic.

This report is presented in 2 parts: (1) abstracts of the scientific papers and discussions by the participants, and (2) new or modified protocols. As the progress achieved suggests that a "vaccine" worthy of pilot clinical trial may be developed within the next year or two, the SWG considered it essential to begin to consider the safety and ethical factors which this would raise. Therefore, these are included in the general report.

(2) FACTORS TO BE CONSIDERED IN ASSESSING THE SAFETY OF MYCOBACTERIAL LEPROSY VACCINES PROPOSED FOR USE IN HUMANS

Careful consideration must be given immediately to the matter of procedures to be followed in assessing the possible toxic effects or other adverse reactions which may be engendered by any proposed mycobacterial leprosy vaccine. The establishment of such procedures is a matter of urgency as highly purified *M. leprae* from armadillo tissue may be available within the coming year for preliminary testing. These procedures will vary in detail depending upon the nature of the proposed vaccine, i.e. whether it be:

(1) killed *M. leprae*;
(2) a killed cultivable mycobacterium;
(3) a live cultivable mycobacterium, or
(4) a combination of one of these with live BCG.

The tests applied will include all those already specified by WHO Expert Committees on Biological and Human Experimentations.

In addition, since both *M. leprae* and cultivable mycobacteria have inherent adjuvant activity, the safety of these proposed vaccines as *adjuvants* will need to be assessed (see *Immunological Adjuvants, Technical Report Series* No. 595, WHO, 1976, pp. 33-35).

In regard to the use of killed *M. leprae* as a vaccine, it is important that the large literature on lepromin testing in humans be examined for reports of adverse reactions which may have been observed.

It is strongly recommended that the IMMLEP Steering Committee, in consultation with the Secretariat of WHO, prepare a detailed testing procedure to be followed for safety evaluation of each of the four types of vaccines listed above, and that it be made available to members of the Scientific Working Group and other interested individuals before 1 December 1977. This will ensure that the preliminary animal tests can begin as soon as a candidate vaccine is selected.

(3) PURIFICATION OF *M. LEPRAE* FROM HOST TISSUES

Dr Draper described modifications to an earlier method (Protocol 2/75) for purifying *M. leprae* (now Protocol 3/77). Bacteria prepared by the new method had unaltered ability to transform lymphocytes (experiments of Dr Gunnar Bjune). Using *M. lepraemurium* as a model, Dr Stanford and Professor Morten Harboe had been unable to find alterations in antigens (measured by immunodiffusion and crossed immunoelectrophoresis respectively) caused by the method of Protocol 2/75 or the modification. Dr Rees and Mrs Celia Lowe, using skin test in guinea pigs, had found very little armadillo material in bacteria prepared by the modified

method. Professor Talwar emphasized the importance of electron microscopy in judging the purity of *M. leprae* preparations, and the possible use of immunoelectron microscopy to detect the presence or loss of bacterial antigens. Dr Lefford, using a model system of BCG in mice, had shown that the method of Protocol 2/75 did not affect viability, but that large losses of bacteria occurred with the discarding of the low-speed sediment.

Dr Nakayama described a method for purifying *M. leprae,* involving countercurrent distribution in an aqueous two-polymer phase system (Annex 1). The method produced pure bacteria to some of which amorphous material adhered. This material came from the large body described as a sporangium (Annex 2), which could be incubated in a special medium to release further *M. leprae.* Dr Kirchheimer pointed out that the name "sporangium" was misleading for a pyocaryotic organism, and Professor Talwar doubted that the amorphous material was of bacterial origin. Dr Nakayama presented a further paper from Dr G. Matsuki and Dr H. Nakagawa (Annex 3), on purification of *M. leprae.*

Dr Juščenko had studied *M. leprae,* prepared according to Protocol 2/75, in the electron microscope (Annex 4) and showed electron micrographs of *M. leprae* and other mycobacteria. He considered that freeze-drying had little effect on the bacteria, but that proteolytic enzymes were rather harmful as judged morphologically. Dr Rees said that viable *M. leprae* sometimes remained after freezing at -70° C; Dr Kirchheimer reported that DOPA oxidase of *M. leprae* from human tissues, but not from armadillo tissues, resisted freezing.

Dr Closs described crossed immunoelectrophoresis experiments to detect antigens from armadillo and *M. leprae* in purified preparations. He proposed the use of the method to standardize antigen preparations, and demonstrated its use to study 4 preparations. Professor Talwar emphasized the need for good antisera if standardization by the method was to be practicable.

Summarizing, Dr Bloom said that the method in Protocol 3/77 seemed to offer improved yields of *M. leprae* with low contamination with armadillo material. Dr Nakayama's interesting 40 μm particles needed further study and their presence *in vivo* should be investigated. It was unwise to describe them as sporangia. Dr Juščenko had shown the value of electron microscopy in studying possible effects of purification procedures (Annex 4). Cross immunoelectrophoresis was a valuable technique for detecting contamination, but for standardization of antigens it presented difficulties in requiring standard antisera. He asked how skin tests in guinea-pigs compared in sensitivity with the immunoelectrophoretic technique; from discussion they appeared to have similar sensitivity.

It was formally agreed that the modifications to Protocol 2/75, included in and now designated Protocol 3/77, should be adopted (for the present) by IMMLEP as a standard method of preparing *M. leprae* suspensions from armadillo tissues.

(4) CELL WALLS

Professor Lederer described the structure of mycobacterial walls, consisting of mycolic acids, arabinogalactan and peptidoglycan. The latter was unusual in the *N*-glycolylmuramic acid replaced *N*-acetylmuramic acid found in other bacteria. Mycobacterial walls were adjuvants, and the activity seemed to reside in the muramyldipeptide portion of the wall (Annex 5). This substance could be synthesized and was commercially available. The effect of variations of structure on adjuvant activity was known. Two other components of mycobacterial walls, cord factor and polyglutamic acid, affected adjuvant activity.

Dr Draper described some analyses of walls of *M. leprae* prepared by the standard Salton process. They resembled other mycobacterial walls but differed in containing glycine and possibly ribose.

Dr Dawidowicz had studied lipid and carbohydrate composition of *M. leprae* walls prepared without using detergents or proteolytic enzymes. They contained fatty acids, apparently straight chain, with 14, 16, 18, 19, 20, 21 and 22 carbon atoms.

Dr Bloom asked whether walls were important in mycobacterial immunology. Dr Shepard said that in the mouse foot-pad, whole *M. leprae* were antigenic while isolated walls were not. Dr Lefford emphasized the importance of the walls of mycobacteria. He pointed out also that proteins, especially serum albumin, bound strongly to walls, of BCG, and that such a preparation was an antigen with a "built-in" adjuvant. Proteins secreted by mycobacteria were similarly presented on the surface.

(5) FRACTIONATION OF ANTIGENS FROM *M. LEPRAE*

Dr Closs reported on work done in Oslo to characterize the *M. leprae* that has been recovered and purified from infected armadillos, using both immunological and non-immunological methods.

(a) *Immunological methods*

Antisera against highly purified *M. leprae* (A10), have been prepared by Dr Draper in 5 rabbits. Contrary to what has been observed when rabbits have been immunized with similar preparations made from other mycobacteria, only a limited number of antibody specificities developed. The antibody response was studied, using crossed immunoelectrophoresis (CIE). The number of precipitin lines that could be detected increased to a maximum level of 6 to 7 lines after 4 immunizations, and did not increase further with prolonged immunization. This is in striking contrast to what is usually observed when making antisera in rabbits against extracts of other mycobacteria, with which it is possible to detect about 40 different antigens in CIE.

The results with *M. leprae* indicate that: (1) the purified *M. leprae* preparation contains less soluble antigens than other mycobacteria used until now, or (2) there may be additional material present which is not immunogenic in rabbits and which is therefore not detectable by their rabbit antisera.

To clarify which of these 2 possibilities is the one more likely to explain the findings in CIE, additional experiments were carried out.

(b) *Non-immunological methods*

Acrylamide gradient gel electrophoresis was performed with 2 different *M. leprae* preparations (A10 and AB14) as well as with sonicates of BCG, *M. smegmatis, M. lepraemurium,* and *M. phlei* made to contain similar amounts of protein as measured by the modified Folin (Lowry) method.

Whereas the extracts made from the 3 cultivable mycobacteria produced between 40 and 50 distinct bands, the A10 preparation produced only 3 very weak bands, and the AB14 preparation produced the same 3 bands but much stronger in addition to one weak band.

Conclusion

By both immunological and non-immunological methods, the extracts made from *M. leprae* purified from infected armadillo tissue apparently contained only about one-tenth of that normally found in extracts made from other mycobacteria.

Dr Closs also reported on work done to investigate the degree to which components specific for *M. leprae* are present in the extracts. This work was done mainly by incorporating antibodies against other mycobacteria in an intermediate gel in the CIE reference system. All the components could be sedimented with several or all of the antisera used, indicating that all the components are widely cross-reacting among mycobacterial species and even Nocardia. The work of Dr Goran Kronvall was briefly mentioned—it has shown that at least one of the components carry *M. leprae* specific determinants.

Dr Dawidowicz reported on work done in Caracas to characterize the components responsible for skin test reactivity. Two different preparations of *M. leprae* were made:

(a) Infected armadillo tissue was disrupted with a Sorvall Omnimixer and the number of acidfast bacilli in the homogenate adjusted to 1.6×10^8 per ml. This suspension was first autoclaved for 15 min and then centrifuged for 2 h at 50,000 rev/min. The supernatant was then filtered through a 0.45 μm millipore filter and used for fractionation studies.

(b) Preparations were also made according to the method described by Dr Draper. The purified bacilli were sonified for 15 min and the sonicate autoclaved.

Preparations (a) and (b) were then run through a Sephadex G-200 column and the various fractions tested for activity in the 48 h skin reaction using patients with tuberculoid leprosy as test subjects. Two active fractions were obtained from both preparations (a) and (b); one with molecular weight greater than 200,000 daltons and another with molecular weight between 8000 and 20,000 daltons. By far the greatest activity was found in the high molecular weight fraction.

Preparation (b) also contained a third peak of material absorbing at 280 nm. However, this material was not active in eliciting a skin reaction in tuberculoid patients.

(6) TAXONOMY

Three papers were read in the taxonomy section.

(a) The first (by J. L. Stanford) stressed the importance of cell-mediated immune processes as a tool for taxonomy. It was pointed out that states of responsiveness and unresponsiveness ("anergy") to skin-test reagents exist in each of the major mycobacterioses. There are 2 types of "anergy" expressed in leprosy. The non-specific type occurring commonly in borderline types of disease has no apparent taxonomic value, but following exclusion of this type, the remaining more specific type of "anergy" predominantly occurring in lepromatous disease has considerable intertaxonomic discriminating power.

Using the technique, *M. marinum, M. nonchromogenicum* and *M. vaccae* show the closest relationship to *M. leprae.* No further work has yet been done with *M. marinum.* Separation of rough from smooth mutants and preparation of separate skin-test reagents from them has made it possible to demonstrate a closer antigenic relationship between the rough strain reagents and LRAB 14 (*M. leprae*), in a recent study carried out in South India.

Preliminary results of tests carried out in guinea-pigs demonstrated the close relationship between *M. vaccae* and *M. leprae* and the systems used might be of potential value in developing a vaccine.

Finally, it was recorded that the organisms grown by Dr Delville were not mycobacteria, but contained typical corynemycolic acids and belonged to the genus *Corynebacterium.* Organisms grown by Professor Skinsnes* were examined by many techniques† and over 95% were found to be *M. marianum* (*scrofulaceum*). The remaining organisms examined under the electron microscope on the basis of size qualified as *M. leprae* but they appeared to be degenerated and no further information about their identity could be obtained. Requests for further material from Professor Skinsnes have not been granted.

(b) Dr Walter presented a hypothesis (Annex 6) suggesting that the use of whole organisms in a suspension similar to lepromin, prepared from various mycobacterial species might provide information of taxonomic value.

(c) Professor Talwar, using a system similar to that proposed by Dr Walter, reported on his study of 71 coded strains. Following selection of 5 of the coded strains by LTT and MIF tests on tuberculoid patients, both Mitsuda and Dharmendra-like reagents were prepared from all 5. These were tested simultaneously in small numbers of tuberculoid and lepromatous patients. All 5 showed a relationship with *M. leprae*-derived reagents, but the number of subjects tested has been insufficient for meaningful studies of correlations. It was agreed that after adequate numbers of persons in each group had been studied, the identity of the 5 strains would be determined and the code broken.

A system for assessment of pathogenicity was proposed, but discussion on it was temporarily postponed. It was recommended that the identity of the selected organisms should be confirmed by laboratories specializing in mycobacterial taxonomy.

It was agreed that Professor Talwar's studies were of the greatest interest and urged that strains should be exchanged between Professor Talwar and Dr Stanford in the near future.

(7) INDUCTION OF CMI TO *M. LEPRAE*

Dr Lefford referred to previous experience with BCG in oil, which had shown that after an early transient swelling at the site of injection, a further phase of swelling developed at 3 to 4 weeks, accompanied by delayed hypersensitivity and immunity to challenge. He had therefore tested doubling concentrations in Tween-saline or water-in-oil emulsions of leprosy bacilli acquired from Dr Rees. Doses from 4 to 1000 μg of bacilli were injected into mouse foot-pads.

---

* Skinsnes, O. K., Matsuo, E., Chang, P. H. C. and Anderson, B. (1975). *In vitro* cultivation of leprosy bacilli on hyaluronic acid based medium. *Int. J. Leprosy* **43,** 193.

† Stanford, J. L., Bird, R., Carswell, J. W., Draper, P., Lowe, C., McDougall, C., McIntyre, G., Pattyn, S. R. and Rees, R. J. W. A study on Skinsnes' leprosy bacillus strain C318,: *Int. J. Leprosy* (in press).

The highest dose gave some initial swelling, which subsequently fell, and the others tended to give a very ill-defined peak at 4 to 6 weeks.

However, when foot-pad tested in the contralateral foot-pad it was found that animals given 16 μg of bacilli or more, showed delayed hypersensitivity to PPD, and resistance to i.v. challenge with 10^5 RIRV. The *M. leprae* organisms given in Tween-saline were as effective or better at inducing these effects than were those given in water-in-oil emulsion. Similarly *M. leprae* in saline was more effective at inducing non-specific resistance to *Listeria* than were either *M. leprae* water-in-oil, or heat-killed BCG.

M. leprae injected in mouse foot-pads in saline also induced delayed hypersentivity to an antigen preparation supplied by Dr Rees' laboratory when tested at 1 month. The optimal test dose was 5 μg.

Dr Shepard reported that both living and heated *M. leprae* injected intradermally into the flanks of mice, caused regional node enlargement, and protection against subsequent challenge with *M. leprae*. BCG was only active if given life. Eleven other organisms were ineffective. The lymph-node enlargement was seen mainly in the paracortical areas.

Mice were immunized with 10^7 *M. leprae* organisms (heat-killed) injected intradermally and foot-pad tests performed at 14 days in the other foot. The optimal eliciting antigen was found to be autoclaved *M. leprae* in PBS or Banks BSS with Tween.

In subsequent series of experiments, all mice were challenged with autoclaved antigen, and the immunizing preparation was varied. Autoclaved antigen also proved best for immunization. This property was unaffected by phenol, or by any of the purification steps in Dr Draper's technique.

Mickle-disintegrated organisms were less effective for immunization whether heated, or unheated. This was true of the whole preparation, and of both cytoplasmic and cell-wall fractions. However, such preparations were effective as eliciting antigens, cell walls being more potent than the cytoplasmic fraction.

Data from an experiment in which all responses were rather low, suggested that Dr Draper's purification steps tended to decrease the efficiency of the organisms as eliciting antigen.

During the discussion, Dr Lagrange suggested that the soluble cytoplasmic fraction might inhibit the induction of CMI by a mechanism analogous to "immuno-deviation".

Dr Shepard noted that lymph-node enlargement caused by killed *M. leprae* appeared to last for 1 year or perhaps for life, in mice. This is not so after killed BCG.

Professor Bloom reported studies in which guinea-pigs had been immunized by injections of 0.5 mg of *M. leprae* organisms supplied by Dr Draper. Sensitization, tested at 1 month was greater when the organisms were injected in aqueous suspension (Hanks BSS), than in suspension in oil (incomplete Freund's adjuvant, without emulsification). The response to 6 μg A14 was greater than to 6 μg PPD. Sensitization also occurred when the *M. leprae* was injected with BCG (10^7), but in these animals PPD reactions were greater than those to A14.

Sonicated organisms sensitized poorly, whether in Hanks or in oil. Organisms suspended in saline and emulsified in adjuvant 65 (containing peanut oil) provided the most effective sensitization of any oil vehicle yet tried.

Antigens prepared from normal armadillo tissues always elicited negative responses. Without *M. leprae,* BCG (10^7) sensitized to PPD, but the A14 response remained negative. Immunization with an *M. vaccae* strain (296R)* in Hanks provided by Dr Stanford, resulted in good positive responses to A14, although less strong than those induced with *M. leprae.*

Intradermal immunization was better than the foot-pad. The animals were retested at 18 weeks. Both diameter and induration were measured. Animals which had received organisms in Hanks gave good responses to A14 by both measurements, whereas, when oil had been used, the diameter of the reaction was large, but induration much less so. At 18 weeks responses in animals given *M. leprae* and BCG were as good or better than those in animals given *M. leprae* only, and animals inoculated with *M. leprae* in emulsion with adjuvant 65 showed larger indurated reactions than when tested at 1 month. When *M. leprae* in Hanks solution (0.1 mg, 0.3 mg and 1 mg) were injected into 4 or 8 intradermal sites, subsequent skin-testing showed no marked dose-response relationship.

---

* This strain differs in a number of respects from those of this species now considered most closely related to *M. leprae.*

In Dr Rees' work, induction of skin-test positivity in guinea-pigs to a number of different batches of *M. leprae,* purified by Dr Draper from armadillo tissues, was used to study the reproducibility of the preparations, and of skin-test antigens prepared from them.

0.5 mg (dry weight) of organisms was injected either as single dose into the groin, or distributed equally in the 4 foot-pads, of guinea-pigs (400 to 300 g Hartley strains). The optimal testing dose of antigen was found to be 6.0 μg. This technique showed there is little variation between preparations.

There were, however, 2 points of disagreement with Professor Bloom's studies.

(1) Organisms in Freund's incomplete adjuvant were more effective than organisms in saline. It was felt that this may have been due to the fact that Dr Rees' organisms were suspended in saline and then emulsified in oil adjuvant while, except for the adjuvant 65 protocol, Professor Bloom's were suspended directly in oil.

(2) In Dr Rees' experiments, *M. leprae*-immunized animals showed some sensitization to PPD. It was felt that this also might be due to differences in physical properties of the adjuvants, or to different populations of environmental organisms in the animal houses.

MDP (Muramyl dipeptide) was nearly as effective an adjuvant (0.2 mg/animal saline with the *M. leprae*) as water-in-oil emulsion.

(8) INDUCTION OF RESISTANCE TO EXPERIMENTAL INFECTIONS OTHER THAN *M. LEPRAE*

Dr Rook presented a paper on the CMI response to a non-pathogenic mycobacterial species, *M. nonchromogenicum* (*Mn*) in mice. CMI was measured in terms of DTH, namely, the 24-h increase in foot-pad thickness following the injection of a cell sonicate of *Mn.* Following 10^8 Mn into the base of the tail, DTH reached a peak at day 11 and decayed rapidly thereafter. This early type of DTH, which was shown to be distinct from the Jones-Mote phenomenon, was evoked by all of 8 mycobacterial species known to be non-pathogenic for the mouse, but not by the pathogens *M. avium, M. ulcerans,* or *M. kansasii.* A recall of DTH could be achieved by re-inoculation of *Mn.* Under these circumstances, the ability to elicit DTH was sustained indefinitely. The hypothesis was advanced that the day 11 evanescent DTH represented the primary antimicrobial effector mechanism and that the induction of a sustained state of high-level DTH was inimical to the host, since it was responsible for the tissue damage and progressive disease characteristic of progressive tuberculosis and tuberculoid leprosy. Furthermore, it was postulated that the induction of transient hypersensitivity was the normal immunological response to a noxious agent which the host could control, and sustained DTH was due to a loss of this normal T-cell regulatory control mechanism induced by virulent parasites. In such cases the host succumbed to infection.

Drs Sansarric, Lagrange, Lefford and Godal participated in the discussion which followed. Lagrange, in particular, was dissatisfied with the evidence on which the main hypothesis was based. Lefford conceded that, while there was no direct relationship between DTH and antimicrobial immunity, the advantage to the host of sustained responses induced by mycobacteria was the generation of a population of memory cells which protected the host for a long period of time. This advantage might well be achieved at the cost of concomitant tissue damage.

Dr Lefford, using a strain of inbred mice (CB6) that was highly susceptible to *Mlm,* found that CMI to that parasite was nonetheless induced following the inoculation of 10^8 live *Mlm* (L-*Mlm*) into the hind foot-pad (HFP). A measure of the induction of CMI was the swelling of the inoculation site (HFP) which commenced at 3 weeks and stabilized at about 6 weeks. In T-cell depleted mice the onset of such swelling was delayed, but then eventually exceeded that of normal mice. A similar suppression of induction of HFP swelling was produced by concomitant i.v. infection with 10^7 to 10^9 *Mlm,* the degree of inhibition being proportional to the dose. The sequence of initial suppression of HFP swelling followed by enhancement of swelling could be produced by concomitant or prior challenge with 10^9 heat-killed (HK) *Mlm* i.v. Attempts were made to reverse the effects of i.v. antigen on the CMI response to *Mlm,* namely, splenectomy and pre-treatment with 10^7 live BCG i.v. Splenectomy was entirely without effect. BCG was unable to reverse the suppressive effect of 10^9 live *Mlnm* i.v., but when given 2 to 4 weeks prior to challenge, ameliorated the effect of 10^9 HK-*Mlm* i.v.

The author concluded that inappropriate methods of immunization were not without hazard, and this should be borne in mind with respect to vaccination against leprosy. There was

some comfort to be derived from the finding that BCG might be able to prevent some of these untoward effects.

In the discussion that followed, Dr Closs pointed out that the apparent enhancement of infection at the site of inoculation might be due to local retention of organisms and the total mouse load of bacilli might not be greater than that of mice that were not challenged intravenously.

Dr Lagrange presented preliminary data from an *Mlm* model system in which he attempted to influence the course of that infection with living BCG. For this purpose both susceptible (C3H) and resistant (C57/bl) mice were used.

The injection of 2×10^7 *Mlm* into the FP produced a progressive increase in FP thickness which eventually plateaued in 2 to 3 months. This response developed more rapidly in C57/B1 than in C3H mice and was shown to be dependent on T-lymphocytes since it did not develop in nu/nu mice. The difference between C57/B1 and C3H was unaffected by varying the size of the infectious inoculum, and was reflected by the recovery of larger numbers of *Mlm* from the popliteal lymph nodes of the latter strain.

Mice were pre-treated with 10^6 live BCG i.v. prior to FP infection with 2×10^7 *Mlm*. BCG treatment did not influence the tempo of FP swelling in C3H mice, but produced a more rapid onset of swelling in C57/Bl mice. The induction of FP swelling by live *Mlm* could be suppressed bu concomitant i.v. injection of either 2×10^8 live or HK-*Mlm* in both mouse strains. Deaths occurred sooner in C57/B1 mice infected i.v. than in C3H mice. Attempts to reverse the suppressive effects of systemic heat-killed *Mlm* with prior i.v. immunization of 10^7 living BCG failed.

The influence of immuno-modulating agents on the induction of immunity to *Mlm* was then examined in B6 x D2 F1 mice. Immunization with HK-*Mlm* either alone or in combination with living BCG and/or cyclophosphamide (CY) was followed by FP infection with live *Mlm*. Pre-immunization with BCG/CY/*Mlm* or BCG/*Mlm* resulted in a DTH response seen 24 h after injection of live *Mlm* which thereafter subsided. Subsequent chronic FP swelling was more rapid in all immunized groups than in controls.

The induction of DTH to *Mlm* was attempted using BCG, CY and CP (*C. parvum*) as modulating agents. Significant sensitivity was induced only by using *Mlm* with BCG or CP, the latter having the greater effect. C57/B1 mice developed greater sensitivity than C3H mice.

Dr Rees presented preliminary data on the induction of immunity to *Mlm* in C3H mice by a combination of living BCG and dead (^{60}Co-irradiated) *Mlm*. Counts of challenge with *Mlm* injected into the foot-pad were available for up to 18 weeks after infection. 10^7 live BCG alone and 10^5 live BCG + 0.001 mg killed *Mlm* appeared to be protective

In the following discussion, Dr Lefford thought the shape of the growth curves suggested that whereas 10^7 BCG alone was exerting a non-specific effect, BCG + *Mlm* appeared to induce a specific immune response. Dr Rees agreed.

(9) RESISTANCE TO EXPERIMENTAL INFECTION WITH *M. LEPRAE*

Dr Shepard presented extensive studies on the protection afforded by various vaccines against mouse foot-pad infection with *M. leprae* (*Ml*). The standard procedure was to inoculate either 10^7 organisms or the equivalent in terms of packed mycobacterial cells intradermally into the flank on day 28. Mice were infected with 5.0×10^3 *Ml* on day 0. The size of the lingual lymph node (draining the immunization site) was monitored at 28-day intervals. When the counts of *Ml* in the FP of controls exceeded 10^6, counts were made from the FP of vaccinated mice. These counts were repeated 90 days later.

The effective vaccines were living BCG (Pasteur/Trudeau and Rosenthal) and dead *Ml*. Other vaccines that had little or no effect included *M. vaccae* (3 strains), *M. nonchromogenicum* (3 strains), live H37a (both IP and intradermal), *Myco. RNA* (Youmans) derived from H37a, *M. phlei, M. smegmatis, M. diernhoferi* and heat-killed BCG. There was a close association between inguinal LN enlargement and protection.

A number of variables affecting the efficacy of *Ml* vaccines was studied. Freshly prepared and frozen-preserved live *Ml* and ^{60}Co-irradiated *Mlm* derived from armadillos were all effective immunogens. However, their efficiency was increased by heating for 30 min at 60° or 80° or autoclaving for 15 min. By contrast, the immunizing efficiency of living BCG was almost entirely lost by heat-killing. Initially it was thought that freeze-thawed *Ml* was also more immunogenic than live *Ml* but this was not confirmed. The influence of the suspending medium was also investigated. Various combinations of PHS/Tween Hanks BSS with or without 10%

BSA were compared. It appeared that there might be a small advantage to using Hanks/Tween. Testing of live *Ml* at different stages of purification revealed no loss of immunogenicity.

In the discussion, Dr Lefford suggested that before the non-*Ml* vaccines were finally discarded as ineffective, much larger inocula should be tried, e.g. 1 to 4 mg dry weight equivalent. Dr Rook indicated that optimal induction of DTH in mice, by a single subcutaneous injection of *M. nonchromogenicum* or *M. vaccae* may require 10^9 organisms, but 10^7 will give a significant response. Alternatively, powerful sustained responses can be induced by a second injection into the same site at 4 weeks. It also emerged that heating *Ml* vaccines resulted in bacterial clumping which might be a factor in immunogenicity.

(10) EPIDEMIOLOGICAL SKIN-TEST STUDIES

The first 2 reports concerned the results obtained under Protocol 5/75 (comparative testing in non-endemic areas). The purpose had been to compare different antigenic preparations from *M. leprae* by skin-testing in non-endemic areas to determine whether there is a correlation with tuberculin sensitivity. More than 60 subjects were to be included in each study. The antigens to be compared were (a) LRA6, a material prepared in Dr Rees' laboratory by disrupting *M. leprae* that had been purified from armadillo livers by Draper's method; (b) LCA, the high-speed centrifugal supernatant from an autoclaved lepromin prepared from armadillo liver; (c) a control preparation from normal armadillo liver, and (d) PPD (RT23, 2 TU).

Mrs Pinardi reported the results she and Dr Convit had obtained. They had carried out the skin-tests in Santiago, Chile. Many of the subjects had been vaccinated with BCG. They found a low percentage (approximately 10%) of positive reaction at 72 h with LRA6 and LCA in healthy adults and tuberculosis patients, and only 0.2% in healthy children. The control armadillo material gave no positive reaction. The PPD gave 92% positive in tuberculosis patients, 68% in healthy adults, and 22% in healthy children. There was suggestive correlation between the reactions from *M. leprae* and those from *M. tuberculosis.*

Dr Rees reported results in the United Kingdom on Oxford students. Most of the students had received BCG vaccine at age 13 to 15. LRA6 had given 8% positive; LCA 8% positive armadillo control only, 61 positive and PPD 38% positive. Most of those reacting to *M. leprae* antigens were positive to PPD, whereas most of those not reacting to *M. leprae* antigens were negative to PPD (Annex 7).

Thus in both studies there was suggestive correlation between the reactions from *M. leprae* and those from *M. tuberculosis,* but many of the positive reactions to *M. leprae* could not be explained as cross-reactions caused by infection with *M. tuberculosis.* However, it should be noted also that there was a 40% (2/5) discrepancy in individuals reacting to both LCA and LRA6.

Mrs Pinardi also described experiments carried out with lepromin from human sources. In one approach, an attempt was made to determine whether the response to a high dose of lepromin (600×10^6 AFB/ml) could be used for determining the need to continue treatment of LL patients who after years of therapy had remained skin-negative. The lepromin site was biopsied 30 days later and showed either absence or persistence of AFB in a macrophage granuloma. If AFB persisted chemotherapy was continued.

She described another study using a "lepromin" test containing one part of BCG vaccine mixed with 9 parts of lepromin (160×10^6 AFB/ml) before injection. In lepromatous patients who were tuberculin positive, a local reaction occurred in which the AFB (including *M. leprae*) were destroyed. *M. leprae* at other sites were not destroyed. In the discussion it was pointed out that the results indicate: (a) local activation of macrophages as a result of interaction between sensitized lymphocytes and BCG, and (b) a lack of immunosuppression by *M. leprae* on the response to BCG.

Dr Stanford than reported the results of his skin-test studies in Burma. Antigen A6, the soluble product after disintegration of *M. leprae* purified by the Draper method, was used as a skin-test antigen in normal controls, leprosy patients, their close contacts, and tuberculosis patients. The incidence of positives was approximately as follows: 20% of controls, 20% of indeterminate, 40% of tuberculoid, 2% of lepromatous patients, and 10% of tuberculosis patients. In close contacts the figure was 8% of females and 38% of males. In other groups the males showed positive rates that were equal to or only slightly in excess of those in females.

Further examination was made of the results in close contacts. In BCG vaccinated, the male: female ratio was 43%:0%, compared to 35%:10% in BCG unvaccinated. In patients with "general anergy" (negativity to 4 other mycobacterial antigens), the ratio was 6%:28% in

vaccinated and 13%:14% in unvaccinated. In patients with "specific anergy" (negativity to *M. leprae*-related antigens) the ratio was 38%:44% and 37%:79% in vaccinated and unvaccinated groups respectively.

The rate of skin-test reactivity to the antigens from 4 selected cultivable mycobacteria was compared in Burma and Uganda. In Burma, BCG vaccine had given much less protection against leprosy than it had in Uganda. The reaction rate was about the same with *M. leprae*-related antigens (those from *M. vaccae* and *M. nonchromogenicum*), but it was much higher in Burma with certain *M. leprae*-unrelated antigens (*M. marianum* and *M. kansasii*) 79% *versus* 3.5% for marianin and 60% *versus* 0% for kansasin. In persons in Burma the incidence of positivity to marianin and kansasin rose from 20 to 30% at age 6 to 60 to 80% at age 15 or more. The results suggested that positivity to marianin inhibited the effect of BCG vaccination in increasing reactivity to the *M. leprae* and related antigens.* Experiments in mice were carried out in England to see if infection by *M. marianum* would inhibit the immune response to BCG. The mice were given *M. vaccae* or *M. marianum* by mouth in the drinking water, and injected with BCG into the foot-pad. Acquisition of tuberculin reactivity was decreased in both orally infected groups, as compared to the group receiving BCG only, especially at a time 11 days after BCG injection. The experiment is continuing.

Specificity and epidemiological significance of the trichloracetic acid precipitated *M. leprae* (armadillo derived) protein prepared by Dr Kirchheimer (Carville) will be studied in India.

(11) IMMLEP *M. LEPRAE* BANK REPORT

Dr Rees submitted a detailed report of the IMMLEP Bank of armadillo-derived *M. leprae* tissues, purified bacteria and antigens held at, and distributed from, London (see Tables 1 and 2). It was gratifying that there was currently in the Bank approximately 4.3 kg of infected tissues as compared with 1.0 kg at the time of the last SWG, December 1975. The tissues received were from 17 armadillos (Carville, 8; GSRI, 4 and London, 5). Allowing for gross fat included with the lymph nodes and skin nodules, the average yield of infected tissues was approximately 210 g per animal. Of the 17 armadillos received since December 1975, 5 (London) had been infected with *M. leprae* from leprosy patients, and the remainder with bacilli passaged in armadillos.

(12) SUPPLY OF *M. LEPRAE*

(a) *Establishment of* M. leprae-*infected armadillos in a non-armadillo area*

The successful establishment of *M. leprae*-infected armadillos in the United Kingdom (housed at the Microbiological Research Establishment, Porton) was presented by Dr Rees (Annex 8). Twenty armadillos were supplied, already laboratory adpated, by Dr Storrs, GSRI, and they were shipped, by air, 5 to a cage, without loss, However, 2 were slightly injured, and one of these subsequently died. No injuries occurred in a recent consignment of 20 armadillos from Florida, caged individually. Dr Rees concluded from his experience and results that it is feasible to establish *M. leprae* infected armadillos in non-armadillo areas of the world. In particular his study showed that a large intravenous dose resulted in a high proportion (11/16) of heavily infected animals surviving 12 to 24 months. The results suggested that the turnover of infected armadillos could be increased by killing-off animals surviving for 2 years.

Dr Kirchheimer, from his much more extensive experience, reported a similar significant increase in the proportion of infected animals by using large intravenous doses of *M. leprae.*

---

* The original hypothesis that the presence of identified leprosy-related species was synergistic with administration of BCG in Uganda and that these organisms were not present in Burma has had to be modified in the light of the results obtained. In view of the evidence that sensitization to the leprosy-related species was almost the same in the 2 countries, an alternative explanation of the fall off in efficiency of BCG against leprosy in Burma was sought. Data was presented indicating that the high level of marianin sensitivity soon acquired with age in Burma might prevent the development of DTH to leprosy and related organisms following vaccination with BCG.

TABLE 1

IMMLEP bank (NIMR: London) of armadillo-derived M. leprae *tissues, purified bacteria and antigen stocks received and distributed in period December 1975-31 January 1977*

tissue bank

Source	Tissues received (g)* Liver	Spleen	Lymph node	Skin	Tissues issued (g)* Liver	Lymph node	Skin	User
Carried forward from 1975					50	–	–	Abe
Carville	375	–	89	–	–	15	–	Convit
GSRI	326	53	85	162	–	17	157	David
Total	701	53	174	162	40	65	–	Rees
					–	169	–	IMMLEP†
Received December 1975–January 1977					90	266	157	
Carville	992	138	200	162				
GSRI	658	84	91	60				
London	800	73	154	286				
Total	2450	295	445	508				
Grand total	3151	348	619	670				

Total in hand 31 January 1977				
Liver	3061	Liver	67	from GSRI dead animals
Spleen	348	Spleen	19	
Lymph node	353	Lymph node	17	
Skin	513	Skin	20	
	4275		123	

*All tissues from killed animals.

†Used for preparing purified bacteria and antigen at NIMR for IMMLEP.

TABLE 2

Purified bacteria and antigen bank

Issued December 1975-31 January 1977 (mg) Purified bacteria	Antigen	User	In hand 31 January 1977 (mg) Purified bacteria	Antigen	Comment
100	1.2	Bloom	909	–	Several batches
160	0.56	Harboe	–	26.8	Several batches
250	1.22	Lefford	–	33.6	Batch LRA6
10	–	Nakayama			
10	–	Ozawa			
50	–	Pervukhin			
–	1.0	Rees			
–	1.0	IMMLEP Protocol 5/75			
580	4.98				

(b) *On the possibility of using a radio-immunoassay in the monitoring of* M. leprae *infection in armadillos*

Dr Closs reported on a study of antibodies against a "common" mycobacterial antigen (*M. leprae* antigen No. 7) in armadillos infected with *M. leprae*. Using a radio-immunoassay technique, higher levels of antibodies were found in animals which at autopsy were found to have established a disseminated *M. leprae* infection, as compared with normal animals. Animals

which had been inoculated but which at autopsy showed no signs of *M. leprae* infection did not have antibody levels differing significantly from those of normal animals.

The method offers the possibility of being able to screen and identify armadillos showing evidence of having encountered infections with mycobacteria. This would be a most important advance in ensuring a "mycobacterial-free" colony of animals for inoculating with *M. leprae*.

(c) *Attempts to increase the supply of* M. leprae

The Chairman of the IMMLEP Steering Committee (Dr Godal), prefaced his report by thanking in particular Dr Kirchheimer for all the infected armadillos he had supplied for the IMMLEP programme at its initiation, when supplies were very short, and also Dr Rees for donating 5 of his infected armadillos to IMMLEP.

Although approaches to several centres where armadillos are found failed to secure new supply centres of infected animals, only one, at the Institute Pasteur de la Guyane française (Dr Baranton), seemed likely to be able to help. However, Dr Godal pointed out that the situation had greatly improved during the past year since it had been established that intravenous inoculation significantly increased the proportion of positive animals (up to 70%) and reduced the time taken to become heavily infected. Therefore, he estimated that the presently established IMMLEP supply centres, together with 8 infected armadillos promised from the US-Japan Leprosy Panel, would provide some 10 kg of infected tissues in 1977.

The Steering Committee had proposed 3 additions to Protocol 1/75 (supply of *M. leprae* for IMMLEP programme):

(i) regular medical examination of all personnel in contact with armadillos;
(ii) rigorous precautions against escape of armadillos from infected area;
(iii) detailed records to be kept of all inoculated armadillos and the records to be available for inspection by WHO.

These additions were unanimously agreed by the SWG, and are incorporated in a new Protocol 1/77 which now supersedes Protocol 1/75.

(13) IMMUNE COMPLEXES

Dr Lambert reported on immune complexes in leprosy sera. With the C_{1q} binding test, increased levels of immune complexes are detected in a high proportion of leprosy patients, especially lepromatous patients, but this test did not distinguish between patients with erythema nodosum leprosum (ENL) and patients without ENL.

On the other hand, increased levels of C3 split products (C3d) have been found only in ENL patients. It is likely that this reflects complement activation in extravascular spaces possibly related to local formation of immune complexes.

A variety of methods is used for detection of immune complexes of which 18 were included in a comparative study organized by WHO. Methods showing promising results in leprosy include the conglutinin binding test, the platelet agglutination test, neutrophil inhibition test and radioactive C_{1q} binding test. For further methodological details see Annex 9.