INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the

SEARO/WPRO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy

Rangoon, Burma, 18–19 November 1981

and the

Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy

New Delhi, India, 14–16 February 1983

Introduction[1]

A scientific meeting on the chemotherapy and immunology of leprosy was held 18–19 November 1981 at the Department of Medical Research, Ministry of Health, Burma in Rangoon, jointly sponsored by the Southeast Asian and Western Pacific Regional Offices of the World Health Organization (WHO) and the immunology of leprosy (IMMLEP) and chemotherapy of leprosy (THELEP) Scientific Working Groups of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. A second meeting, devoted to the immunology and epidemiology of leprosy, was jointly sponsored by the Indian government, Indian Council for Medical Research, and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

The first presentations reviewed the status of chemotherapy in leprosy and are to be published in *Leprosy Review*. Dr. L. Levy described the goals of the THELEP program and the strategy that has been adopted to achieve these goals, and particularly controlled clinical trials of the chemotherapy of lepromatous leprosy. Dr. M. F. R. Waters discussed the background of the trials, sketching the historical development of the concept of trials in which the attempt is made to detect "persisting" *Mycobacterium leprae* by innoculation of immunosuppressed mice and describing the design of the trials. Dr. R. J. W. Rees presented the available results from the innoculation of mice, with particular emphasis on the unexpectedly high prevalence of primary resistance to dapsone in both the Central Leprosy Teaching and Research Institute, Chingleput, South India, and the Institut Marchoux, Bamako, Mali, sites of the current trials. Dr. S. R. Pattyn described the design of the THELEP field trials of chemotherapy in lepromatous leprosy in which lepromatous patients are being treated with largely intermittent, multidrug regimens for two years after achieving smear negativity, following which chemotherapy will be stopped and relapse rates measured. This presentation was followed by a description of the SEARO-sponsored Burma rifampin trial by Dr. Maung Maung Ghi. This trial involved an attempt to interrupt transmission of *M. leprae* from infectious sources to contacts by the addition of a brief (two-week) course of daily rifampin to the standard dapsone monotherapy; the hoped for result is a decreased attack rate, compared to that in the control population, in which only standard dapsone monotherapy is em-

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

ployed. Dr. G. A. Ellard then discussed the problem of the generally poor compliance of leprosy patients with prescribed chemotherapy.

There followed several papers on various aspects of resistance to dapsone which revealed the magnitude of the problem for the first time. Dr. J. M. H. Pearson presented the problem as it was understood at the time THELEP activities began, and described the strategies adopted by THELEP to further elucidate the problem. Dr. M. C. Christian presented an up-to-date summary of the continuing prevalence survey of secondary dapsone resistance in Gudiyatham Taluk, South India. Dr. R. S. Guinto presented the results of the recently completed survey of primary resistance to dapsone in Cebu, The Philippines. Dr. P. N. Neelan and Dr. Kyaw Lwin described the ongoing survey of secondary resistance in Trivellore Taluk, South India, and Myingyan Township, Burma, respectively. Dr. Ji Baohong presented the results of a virtually completed survey of secondary resistance in Shanghai Municipality. Finally, Dr. Robert Utji presented the first results of an informal survey of dapsone resistance in Jakarta, including a case of primary resistance. It was clear from these presentations that the rate of increase in both secondary and primary resistance to dapsone was increasing more than anticipated, which presents a pressing challenge to new strategies for chemotherapy.

It was in this context that the scientific basis for immunological intervention in leprosy was discussed, the papers for which are published here. The research plans and progress of the IMMLEP program were summarized in presentations by Drs. Bloom, Godal, Rees, Shepard, and Buchanan. Dr. Bloom discussed rationales for vaccination against leprosy; Dr. Godal reviewed immunological mechanisms in leprosy; Dr. Rees discussed the production of *M. leprae* from armadillos, the IMMLEP Bank for *M. leprae*, and the purification procedure; Dr. Shepard presented results of animal vaccination studies with various preparations, and discussed the use of animal models for studying immunological unresponsiveness to *M. leprae*; and Dr. Buchanan discussed the use of monoclonal antibodies and development of sero-epidemiologic methods. These basic presentations were followed by three papers involving studies carried out on human beings with potential "vaccine" preparations. Dr. Convit presented his data on immunotherapy on a large number of lepromatous and borderline patients with a mixture of killed *M. leprae* and live BCG. Dr. Deo reviewed data on studies carried out by his group on patients using the ICRC bacillus. Dr. Talwar presented his data on human studies using *Mycobacterium w* and also a preparation of hapten-modified *M. leprae*.

Following the presentation of preliminary human sensitization studies, Dr. Fine reviewed the epidemiological considerations relevant to leprosy, and Dr. Nordeen discussed the epidemiological problem involved in vaccine trails. The operational problems in vaccine trails were presented by Dr. Guld, particularly in relation to the experience in the BCG trails in South India.

In addition to the formal presentations included here, there followed a round-table discussion on epidemiological studies in leprosy in which the various field studies being carried out by scientists from endemic countries were discussed, as well as opportunities for their participation in IMMLEP/THELEP-supported programs.

Participating in the November 1981 meeting were:

Dr. M. Abe
National Institute for Leprosy Research
Tokyo, Japan

Dr. A. B. Adiga
Ministry of Health
Pachali, Nepal

Dr. Anan C. Pakdi
SEARO
New Delhi, India

Dr. Aung Win Thien
Department of Medical Research
Rangoon, Burma

Dr. Ayele Belehu
Armauer Hansen Research Institute
Addis Ababa, Ethiopia

Dr. Bencha Petchelai
Ramathibodi Hospital
Bangkok, Thailand

Dr. V. N. Bhatia
Central Leprosy Teaching and Research Institute
Chingleput, South India

Dr. B. R. Bloom
Albert Einstein College of Medicine
Bronx, New York, U.S.A.

Dr. Vicharn Vithayasai
Chiang Mai University
Chiang Mai, Thailand

Dr. M. F. R. Waters
Hospital for Tropical Diseases
London, England

Dr. Ye Gan-yun
Chinese Academy of Medical Sciences
Taizhou, China

Dr. Y. Yuasa
Sasakawa Memorial Health Foundation
Tokyo, Japan

Participants in the February 1983 meeting included:

Dr. B. A. Askonas
National Institute for Medical Research
London, England

Dr. B. R. Bloom
Albert Einstein College of Medicine
Bronx, New York, U.S.A.

Dr. T. M. Buchanan*
University of Washington
Seattle, Washington, U.S.A.

Dr. M. Christian
Schieffelin Leprosy Research and Training Centre
Karigiri, India

Dr. G. W. Comstock
The Johns Hopkins University School of Hygiene and Public Health
Washington County Health Department
Hagerstown, Maryland, U.S.A.

Dr. J. Convit*
Instituto Nacional de Dermatologia
Caracas, Venezuela

Dr. M. G. Deo
Cancer Research Institute (Tata Memorial Centre)
Parel, Bombay, India

Dr. K. V. Desikan
Central JALMA Institute for Leprosy
Agra, India

Dr. P. E. M. Fine
London School of Hygiene and Tropical Medicine
London, England

Dr. T. Godal
The Radium Hospital
Montebello, Norway

Dr. M. D. Gupte
Department of Social and Preventive Medicine
Mahatma Gandhi Institute of Medical Sciences
Sevagram, District Wardha, India

Dr. R. K. Mutatkar
Department of Anthropology
University of Poona
Pune, India

* Unable to attend.

Dr. P. N. Neelan
Central Leprosy Teaching and Research Institute
Chingleput, India

Dr. M. Pinto
Department of Microbiology
University of Peradeniya
Peradeniya, Sri Lanka

Dr. V. Ramalingaswami
Indian Council for Medical Research
New Delhi, India

Dr. N. M. Samuel
The Leprosy Mission
Anandaban Hospital
Kathmandu, Nepal

Dr. C. C. Shepard
Leprosy Laboratory
General Epidemiology Branch
Bacterial Diseases Division
Centers for Disease Control
Atlanta, Georgia, U.S.A.

Dr. G. P. Talwar
Department of Biochemistry
All India Institute of Medical Sciences
New Delhi, India

Dr. S. P. Tripathy
Tuberculosis Research Centre
Chetput, Madras, India

Dr. C. M. Vellut
Hemerijckx Leprosy Centre
Tamil Nadu, India

Dr. Das Gupta
Deputy Drugs Controller (India)
DGHS, Nirman Bhawan
New Delhi, India

Dr. K. K. Koticha
Medical Superintendent
Leprosy Hospital
Bombay, India

Dr. V. Sen Gupta
Central JALMA Institute for Leprosy
Agra, India

Dr. D. B. Bisht
Additional Director-General of Health Services
DGHS, Nirman Bhawan
New Delhi, India

Dr. K. C. Das
Assistant Director-General (Leprosy)
DGHS, Nirman Bhawan
New Delhi, India

Dr. Gangadhar Sharma
State Leprosy Officer
Tamil Nadu, Madras, India

Dr. G. Ramu
Central JALMA Institute for Leprosy
Agra, India

Dr. Indira Nath
All-India Institute of Medical Sciences
New Delhi, India

Secretariat

Dr. S. K. Noordeen
Secretary
IMMLEP and THELEP Steering Committees
Leprosy Unit
WHO Headquarters
Geneva, Switzerland

Dr. H. Sansarricq
Chief, Leprosy Unit
WHO Headquarters
Geneva, Switzerland

Dr. G. Torrigiani
Chief, Immunology Unit
World Health Organization
Geneva, Switzerland

Dr. T. Matsushima
Medical Research Officer (Tropical Diseases)
WHO/SEARO
New Delhi, India

Dr. N. K. Shah
Regional Adviser
Communicable Diseases
WHO/SEARO
New Delhi, India

Dr. B. B. Gaitonde
Laboratory Services
WHO/SEARO
New Delhi, India

Dr. Anan C. Pakdi
Medical Officer (Leprosy)
WHO/SEARO
New Delhi, India

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Rationales for Vaccines Against Leprosy[1]

Barry R. Bloom[2]

The IMMLEP program of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases has as its goal to develop a vaccine against leprosy. The IMMLEP program was founded on the premise that it would be possible to develop a vaccine which provided protection against clinical leprosy. The availability of *Mycobacterium leprae* grown in the armadillo and methods developed for purification of bacilli from armadillo tissue made the possibility feasible.

Correlation between specific cell-mediated immunity and resistance

The basic assumption of any vaccine is that induction of a state of immunologic reactivity to *M. leprae* antigens will lead to protection. Perhaps the key finding that establishes a relationship between immunity and protection derives from the observation of the different courses of disease across the spectrum of leprosy. It is clear that BT and TT patients express strong levels of cell-mediated immunity and have the capability of restricting the growth of *M. leprae*, although this process may cause tissue damage and clinical problems. LL and BL patients are less able or unable to restrict the growth of the organisms and lack cell-mediated immunity. In contrast, there appears to be a negative correlation between the level of circulating antibodies in patients and the ability to restrict the growth of *M. leprae*, higher titers generally being found in lepromatous than in tuberculoid patients. The basic premise, then, is supported by a strong correlation between cell-mediated immunity and the ability of patients to kill or restrict the growth of *M. leprae*. That is simply a correlation, however, not a proof or a guarantee that a person exhibiting cell-mediated immunity to leprosy bacilli cannot develop clinical leprosy.

A second line of evidence that supports this approach is the observation of Dharmendra and Chatterjee (3) that lepromin-positive individuals who develop leprosy never develop the lepromatous form; they only become borderline or tuberculoid. The correlation between cell-mediated immunity and the resistance to growth of the organism in tuberculoid patients and the correlation between cell-mediated immunity macrophage activation and increased cytocidal oxygen metabolites and degradative enzymes suggests that induction of cell-mediated immunity should lead to increased resistance, although that resistance cannot be conceived of as being absolute.

Specific cell-mediated immunity can be induced with killed *M. leprae* or other mycobacteria

The second specific experimental premise is that *M. leprae* or other cultivatable mycobacteria can produce cell-mediated immunity to antigens of the leprosy bacillus. Probably the first line of evidence to support this view is a modern reinterpretation of the Mitsuda test for leprosy. The Mitsuda test would appear to be unique among all tests for cell-mediated immunity in that it is read not at 24–48 hr but at 28 days. Since in almost all other systems it is possible to detect pre-existing immunity by skin tests that are read at 48 hr, an alternative to the traditional interpretation of the Mitsuda test is that it is not only a skin test which measures pre-existing cell-mediated immunity but is, in fact, a minimal vaccine. As such,

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] B. R. Bloom, Ph.D., Professor and Chairman, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461, U.S.A. Chairman, IMMLEP Steering Committee, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

it has been designed to discriminate between individuals who are unresponsive to antigens of the leprosy bacillus (either because they have lepromatous disease or because they have been unexposed to the bacillus or crossreactive antigens) and those who have already been clinically or subclinically infected and for whom the Mitsuda test is a "booster" which augments weak, prior-existing sensitization or, in fact, simply is able to sensitize in 28 days. The fact that a significant percentage of normal individuals in leprosy nonendemic countries or areas become Mitsuda positive suggests either that it is a weak vaccine or that some individuals have been primed against crossreactive antigens.

A second line of evidence indicating that *M. leprae* is immunogenic derives from the studies of purified *M. leprae* carried out in mice, guinea pigs, and armadillos which indicate that, even in the absence of any adjuvants, purified and killed *M. leprae* are capable of engendering delayed-type hypersensitivity. In the mouse and in the armadillo there is clear evidence that protection against *M. leprae* infection is induced as well. On the other hand, the specificity remains unclear. At present, using monoclonal antibodies it appears that there will be specific antigenic determinants that are specific to the leprosy bacillus. While there are unique epitopes on some protein and glycoprotein antigens, there are other crossreactive determinants on the same molecules, suggesting that there is no unique species of protein. The only unique species of antigen appears to be the phenolic glycolipid I antigen of Brennan. It is clear that vaccination of mice with BCG will protect against growth and dissemination of live *M. leprae*, and there is clear evidence that sensitization with *M. leprae* will lead to crossreactions with a variety of other mycobacterial antigens. It is thus very difficult to establish what the unique and specific antigens of the leprosy bacillus are, whether some must be included in the vaccine to induce protection, whether other antigens will induce unresponsiveness, and whether other mycobacteria share these key antigens. The dilemma in interpreting crossreactive immunization is compounded by the results of two large-scale BCG vaccination trials in which the degree of protection against leprosy initially varied from 80% in Uganda to 20% in Burma. The reasons for the difference in results remain unknown.

Vaccine strategies

There are at present two rationales for vaccination against leprosy. One is immunoprophylaxis, which is designed to protect a population at risk against developing clinical leprosy. The second is immunotherapy, which is designed to convert anergic lepromatous patients to a state of specific cell-mediated immunity in the hope that they will then cure their infection and, ultimately, their disease.

A killed *M. leprae* vaccine. Such a vaccine would be designed exclusively for immunoprophylaxis, since a vast amount of evidence indicates that lepromatous patients are immunologically unresponsive to the leprosy bacilli they are harboring and to *M. leprae* antigens introduced in skin tests. The premise would be that a naive population would be primed to positive immune reactivity to specific antigens of *M. leprae*. When they became infected at some later time, the infecting organisms would serve to boost their already existing levels of cell-mediated immunity and the patients would develop either subclinical leprosy and eliminate the organisms or, at the worst, would develop a mild, tuberculoid-type, self-healing disease.

Such a vaccine has the potential for providing information on one of the key problems in leprosy, namely, the identification of patients at high risk for lepromatous leprosy. Nonresponders to repeated vaccination could be considered immunologically unresponsive and at high risk, identified, and treated with chemotherapy, just as was done in tuberculosis in some countries.

Killed or live cultivable mycobacterial vaccines to provide crossreactive immunity against *M. leprae*. The first experimental tests of this strategy were those using a BCG vaccination for protection against leprosy where the results in different parts of the world yielded vastly different rates of protection. There are studies of small numbers of patients with borderline or polar lepromatous leprosy who were vaccinated with BCG in which clinical improve-

ment was reported, although many of the patients developed reversal reaction symptoms. Preliminary studies on the efficacy of cultivated mycobacterial vaccines in producing immunological conversion in lepromatous patients have recently been very encouraging ([3]), but there are potential difficulties. The first is that in the absence of identifiable *M. leprae*-specific antigens, it is very difficult to know which mycobacteria have appropriate specific antigens cross-reactive with antigens required for protection against *M. leprae*. In this regard, the ability to produce continuous antigen-specific human T cell lines may provide a unique approach to defining determinants which may be important in cell-mediated immunity. A second concern is that even if unique specific or crossreactive antigens are found, how can one be sure that they will not engender immunological unresponsiveness or suppression, rather than priming for immunity to the key antigens? (A question which also could be asked of the killed *M. leprae* vaccine itself.) A third concern is the use of living microorganisms in people who may have some immunodeficiency or immunological unresponsiveness against mycobacterial antigens. One of the appealing aspects of this strategy, however, is the ability to produce very large amounts of such a crossreactive vaccine very inexpensively.

A vaccine of killed *M. leprae* plus living BCG. The basis for this vaccine is derived from Dr. Convit's observations ([2]) that when killed *M. leprae* were injected into the skin of lepromatous patients together with BCG, there was degradation and clearance of the *M. leprae*, which was not seen when the leprosy bacilli were inoculated alone. Based on these observations, Convit has demonstrated that such a vaccine of killed *M. leprae* plus BCG has strong immunotherapeutic effectiveness in patients with indeterminate and borderline lesions, and most recently with polar lepromatous leprosy, leading to skin test conversion, degradation of organisms in the skin, and marked clinical improvement ([1]), although the immunological rationale for this mixed vaccine remains to be elucidated. In any case, the data available on several hundred patients clearly indicate that this vaccine has therapeutic efficacy in many patients who are otherwise anergic, and should have immunoprophylactic potential in the normal population. One advantage of this vaccine would be that if there were patients at high risk for lepromatous leprosy or harboring leprosy bacilli, this vaccine may well force them to immunoconversion and therefore serve therapeutically to cure their infection while it is still subclinical.

Problems inherent in vaccines against leprosy

Epidemologic. The only way that any of these vaccine strategies can be evaluated ethically and meaningfully is by first inducing resistance in appropriate animal models and then by field trials in man. Many animal studies with purified *M. leprae* have already been done or are in progress. Relatively small-scale field trials can be set up to ask the question whether these antigen preparations are capable of inducing cell-mediated immunity to antigens of the leprosy bacillus. It becomes a much greater problem to ascertain whether induction of cell-mediated immunity confers with it resistance to infection by *M. leprae*. For therapeutic trials, which in this case become the most feasible, one simply has to test relatively small numbers of patients with well-defined stages of leprosy and look for therapeutic benefits as well as immunoconversion. With respect to protection of normal populations, field trials become very complex. *M. leprae* are very slow-growing organisms, the prevalence rate may be as low as 0.5 per 1000 population, and assuming that four out of five cases of leprosy are likely to be of the tuberculoid variety, this means that one may have to vaccinate 1000 people to see a diminution in one detectable case of lepromatous disease over a decade. The third population for vaccination which is appealing is that of household contacts of patients with lepromatous leprosy, who are known to have a higher incidence of leprosy, yet the logistics of identifying those individuals, vaccinating, and monitoring them are probably more cumbersome than larger scale mass vaccinations in field trial areas in many countries.

There is basically no precedent for using a vaccine against a disease of such long duration and low prevalence, and one must assume that the study would have to be con-

tinued for 10–15 years before results could be evaluated.

Ethical considerations. In order for any vaccine to be recommended for field trials it must be shown to be safe and effective in small-scale studies. There is a vast amount of data from Mitsuda testing that indicates that killed *M. leprae* does not pose any substantial risk to humans, and one expects purified bacilli to be even more free of toxicity. Even there, the maximal dose required for sensitization and for determining patient acceptability has yet to be established. The second consideration is whether or not there is evidence in animals that the vaccine is protective against *M. leprae* infection. We know that both components of the Convit vaccine, killed *M. leprae* and live BCG, are protective in mice against infection with freshly isolated human bacilli. With respect to vaccines using cultivable mycobacteria, it is important to establish that they have some protective activity against *M. leprae* infection in an animal model to provide the kind of justification required by the World Health Organization (WHO) for sanctioning its use in man.

"The vaccine causes leprosy." One prediction in vaccinating a large population in a leprosy endemic area is a likelihood that patients who have indeterminate or borderline disease, without having manifested clinical symptoms, will begin to show the signs of tuberculoid leprosy after vaccination. There are two consequences. The first is that one can almost certainly expect the cry from the public health and administrative authorities that the vaccine is causing disease, and it will take a long process of education and preparation as well as careful monitoring and availability of appropriate treatment to minimize this problem. The more serious consequence is that some patients who are harboring the leprosy bacillus around the nerves, as they develop rapid cell-mediated immunity, may be expected to develop nerve damage. This must be anticipated, and appropriate and rapid treatment provided. It is encouraging that only a very small number of Convit's vaccinated patients developed any neurologic symptoms, and they were no more severe than those found with chemotherapy.

The duration of sensitization. Because of the low incidence of leprosy, and the long latent period before the disease is manifest, it is necessary that such a vaccine have enduring sensitization in order to provide protection over a long period of time. The duration of sensitization in man remains to be established. If a vaccine is not able to confer high levels of sensitization over a ten-year period, then revaccination or booster vaccination of the population may have to be considered in any vaccine protocol.

Specificity of the vaccine. From a scientific point of view, it is important to establish which of the potential vaccination strategies is the most effective. This requires a comparison of any of the strategies listed above with, for example, a BCG vaccine trial, and with each other. In essence, then, if this were a laboratory experiment it would be a trial with at least four experimental arms and one unvaccinated arm. It is clearly not feasible, either in terms of the availability of populations or financial support for such trials in the foreseeable future, to carry out five-armed studies over this long a period of time. It is unfortunate that difficult and possibly arbitrary or pragmatic choices will have to be made.

Unknown variables. While a great deal of information is available, there remains a large number of scientific variables that will not be understood at the time of vaccination trials, including: a) the mode of transmission; b) what factors determine the form of disease; and c) what role environmental mycobacteria have in enhancing or suppressing responsiveness of different populations to the vaccine. Yet each of these could play a significant role in determining the outcome in an individual and in a population.

In this regard, it is my view that any proposed vaccine trials must be undertaken initially as small-scale field studies with defined questions, which can be expanded stepwise in a phased schedule to encompass more individuals and to ascertain if the immunological efficiency is high and the risks and side effects are low. As these phased studies continue, at some point it becomes possible to begin to assess protective efficacy and, hence, these can truly be considered a vaccine trial.

Prospects for vaccines

In light of the difficulties and unknowns, why entertain the possibility of a vaccine

against leprosy? We believe there are three key reasons. With what is already known from the basic immunological studies and therapeutic vaccine trials in leprosy patients, it seems possible to produce cell-mediated immunity and some degree of protection against the leprosy bacillus. The materials for many of these vaccine strategies are either currently available or likely to become available within the next few years. This makes the vaccine strategy a feasible one. Knowledge of basic immunologic mechanisms operative in resistance to infection and the identification of specific antigens of the leprosy bacillus will be expanding at a much more rapid pace than will the results of clinical trials. This makes the issue an urgent one. Finally, leprosy is a unique scourge of man from historical times to the present, and engenders a unique kind of fear because it is a disease of the mind as well as the body. Since there is no obvious animal reservoir of the disease likely to be involved in transmission to man, the prospect is that a successful vaccine has the potential to eradicate leprosy from the face of the earth in one generation or less. This, plus the obvious limitation of chemotherapy, including the limited epidemiological effectiveness of treating people who already have disease, the logistical difficulties of, for example, case finding, and the emergence of drug-resistant organisms make the case for a leprosy vaccine a compelling one.

REFERENCES

1. Convit, J., Aranzazu, N., Pinardi, M. E. and Ulrich, M. Immunological changes observed in indeterminate and lepromatous leprosy patients and Mitsuda-negative contacts after the inoculation of a mixture of *Mycobacterium leprae* and BCG. Clin. Exp. Immunol. **36** (1979) 214–220.
2. Convit, J., Pinardi, M. E., Rodriguez-Ochoa, G., Ulrich, M., Avila, J. L. and Goihman, M. Elimination of *Mycobacterium leprae* subsequent to *in vivo* activation of macrophages in lepromatous leprosy by other mycobacteria. Clin. Exp. Immunol. **17** (1974) 261–265.
3. Dharmendra and Chatterjee, K. R. Prognostic value of the lepromin test in contacts of leprosy cases. Lepr. India **27** (1955) 149–152.

INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Impressions on the Expression of Suppression in Leprosy[1]

Tore Godal, Abu Salim Mustafa, and Abebe Haregewoin[2]

This paper will focus on our present understanding of immunological mechanisms involved in lepromatous leprosy and the lepromin reaction.

Lepromatous leprosy

Several mechanisms have been proposed during the last two decades to explain the immunological defect in lepromatous leprosy: a) nonspecific anergy, b) defect of antigen-presenting cells, c) immunological enhancement (T cell blocking by antibody at the "peripheral" level), d) immunological tolerance (clonal deletion), and e) suppression.

The last three mechanisms all have in common that T cells play a central role in the deficiency, although the contribution of antigen presentation mechanisms (b) in the pathogenesis remains unclear. In the latter half of the 1960s, nonspecific immunodeficiencies were a popular concept in immunology. With the interest in tumor immunology in the early 1970s, the blocking of T cell function by antibody was thought of as a mechanism applicable to a number of conditions, including leprosy, but as reviewed elsewhere ([8]), both mechanisms appear to be unlikely. The T cell deficiency in lepromatous leprosy shows specificity to *Mycobacterium leprae* antigens and cannot be blocked by humoral antibody *in vitro* and, thus, lepromatous leprosy was found to have features in common with immunological tolerance or "central failure." This does not mean that nonspecific defects do not occur, but patients with such defects may die quickly from other diseases, such as viral infections. The specific defect(s), therefore, may result from a selective process.

Then came the discovery of suppressor cells—the main concept being explored at present. From a theoretical point of view, it is presently difficult to envisage that any state of specific immunological unresponsiveness can be due to any mechanism other than suppression. The reason for this is as follows: The molecular biology of immunoglobulin synthesis is now known in some detail due to recent advances in deoxyribonucleic acid (DNA) technology. A point that has emerged from these studies is that the specificity of the antibody molecule is determined by a random process at the DNA level before cells have acquired antibody molecules (receptors) at the cell surface ([10]). This means that we have pre-receptor B cells with DNA that can code for antibody for any antigen, including antigens of our body. Thus, various kinds of immunological nonresponsiveness, including immunological tolerance, must involve one type of suppression or another. Many suppressor circuits have been uncovered which are involved in this regulation. They may be antigen specific, idiotype specific, or nonspecific, restricted or nonrestricted with regard to the major histocompatibility system ([5]). Studies in this field may therefore require that meticulous attention be paid to methodology. Moreover, not all suppressor pathways are likely to be important in the pathogenesis of lepromatous leprosy.

Many details of how T cells effectuate immunity to intracellular organisms like *M. leprae* remain unclear, but evidence is

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] T. Godal, M. D., Ph.D., M.Sci., and A. S. Mustafa, Ph.D., Laboratory for Immunology, Department of Pathology and the Norwegian Cancer Society Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway. A. Haregewoin, M.D., Armauer Hansen Research Institute (AHRI), P.O. Box 1005, Addis Ababa, Ethiopia.

THE TABLE. *Some characteristics of suppressor cells detected in healthy subjects and multibacillary patients.*

	Current study	Bloom and Mehra [8]
Inducing antigen	BCG/PPD	*M. leprae* (Dharmendra)
Antigen specific in expression	Yes	No
MHC restricted in expression	Yes	No
T cell subset	T4	T8
Disease related	No[a]	Yes[b]

[a] Found in healthy subjects.
[b] Found only in BT-LL patients, but not in TT or healthy subjects.

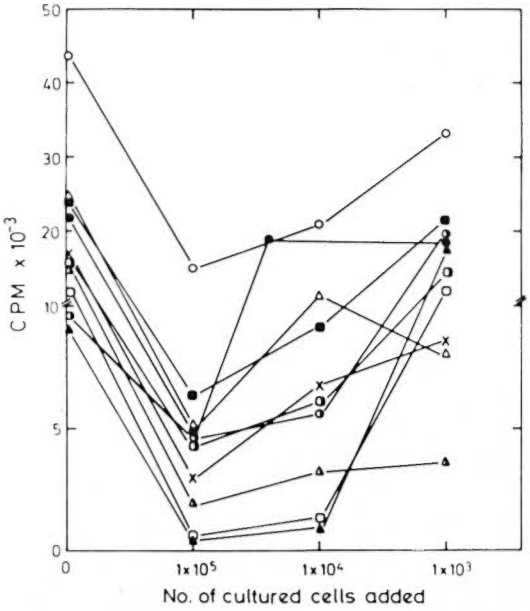

FIG. 1. Effect of BCG induced suppressor cells on the proliferative response of fresh autologous cells. PBMC were obtained from the blood of normal subjects by Ficoll-Hypaque density centrifugation. Primary cultures for the induction of suppressor cells were set up in plastic flasks. In a humidified atmosphere of 5% CO_2, 1×10^6 cells/ml RPMI 1640 with 10% AB serum were cultured with BCG (20 μg/ml) for 5 days at 37°C. After 5 days of incubation, cells from the primary cultures were washed 3 times with RPMI 1640. Secondary cultures were set in 96-well microtiter plates, and 1×10^5 fresh cells were seeded in each well. Graded numbers of precultured cells were added to some of the wells with fresh cells. An optimal concentration of BCG (20 μg/ml) was added to individual cultures. Plates were incubated in a humidified incubator with 5% CO_2 at 37°C for 5 days. Each well received 1.25 μCi of ^{3}H-thymidine 4 hr prior to harvest. Cultures were harvested with a Skatron multiple cell harvester (Lierbyen, Norway). Radioactivity incorporated was measured in a Beckman liquid scintillation counter. Results are given in terms of counts per minute (cpm). Median values from triplicates were used for the expression of the data from individual experiments.

steadily accumulating that T cells, by releasing migration inhibition factor (MIF) and other soluble factors (often called lymphokines, but likely in the future to be included in the Interleukin group as the factors become purified and biochemically defined[1]), may activate macrophages which then kill (or prevent multiplication of) the parasite, e.g., mycobacterium, residing inside them via oxygen metabolites ([15]). However, resistance *in vivo* is obviously more complex, being dependent not only on the local activation of macrophages but also on their mobilization, i.e., recruitment into the local site, as clearly outlined by Mackaness ([13, 14]).

The study of suppressor cells in leprosy has been carried out by various groups with very divergent results ([3]). The explanation of these divergent results remains unclear, but it appears likely that technical differences, limitations in the present technology, and the complexity of mechanisms involved in the regulation of the immune system may explain the results obtained.

This point may be illustrated by the different results obtained by us with BCG in normal subjects as compared to the data obtained by Bloom and Mehra ([3]) in multibacillary leprosy patients.

As shown in Figure 1, we have been able to demonstrate suppressor cells *in vitro* to BCG in purified protein derivative (PPD) positive healthy subjects. This suppression is pronounced when "suppressor cells" are added to fresh cells in a ratio 1:1, and can also be clearly observed at a tenfold lower concentration. Further studies on this suppressor effect indicate that it carried antigen specificity, is MHC restricted, and mediated by T4 cells. The contrasting characteristics between these cells and those described by Bloom and Mehra ([3]) are outlined in The Table.

These differences suggest that in the fur-

ther dissection of the immunological deficiency in lepromatous leprosy, the question is not suppression or nonsuppression, but what kind of suppression. Is there any relationship between these? As outlined by Germain and Benacerraf ([5]), they may represent different stages along a common ("concensus") suppressor pathway. We have recently also embarked on an alternative approach to the study of the defect in lepromatous leprosy. Numerous studies recently have shown that T cells with functional capabilities after initial triggering with antigen can be maintained in a state of continuous proliferation *in vitro* when cultured in medium containing Interleukin 2 (IL-2) ([2,6,7,11]). Thus, we were interested to see if the proliferative unresponsiveness of lepromatous T cells could be due to a lack of IL-2 or related factors by adding exogenous IL-2 rich, T cell conditioned medium (TCM) with *M. leprae* to lepromatous peripheral blood mononuclear cells (PBMC). The results of these studies ([9]) show that lepromatous T cells fail to produce IL-2 after exposure to *M. leprae* and may respond by proliferation to *M. leprae* in the presence of TCM (Fig. 2), suggesting that the unresponsiveness in lepromatous leprosy commonly is due to a deficiency in the production of IL-2 and related factors and not to a lack of *M. leprae*-reactive T cells.

A major point in this area of research is to try to distinguish between those phenomena that are secondary to antigenic load and those that play a primary role in the pathogenesis of lepromatous leprosy. In considering this point, we may turn to the lepromin reaction.

The lepromin reaction

With regard to the late lepromin reaction, significant progress has been made during the last few years through the IMMLEP program. Firstly, due to the availability of abundant quantities of highly purified *M. leprae*, a refined and standardized preparation of *M. leprae* has become available. Secondly, due to standardized soluble skin test preparations, which have also become available through IMMLEP, by which sensitization to *M. leprae* can be studied by skin testing. Because of crossreactivity, this can at present only be done in selected groups

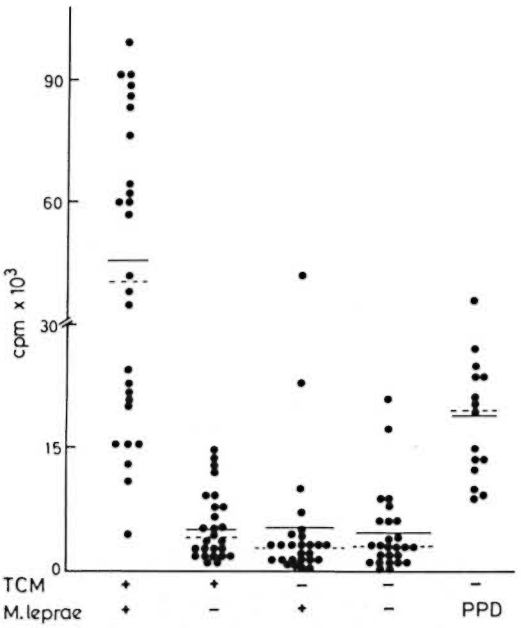

FIG. 2. PBMC were prepared from defibrinated venous blood by centrifugation on Ficoll-Isopaque. In 96-well, round-bottomed trays (Linbro) 0.2 × 10^6 cells were cultured in triplicate in 200 μl of RPMI 1640 medium containing 20% pooled, heat-inactivated, normal human serum; 2 mmol/l l-glutamine and antibiotics. Test cultures contained 25 μl of whole washed *M. leprae* with a concentration of 3 × 10^7 bacilli/ml, which gives optimal proliferation in tuberculoid patients or PPD (10 μl of 100 μg/ml). To the cultures were added 100 μl/well of T cell conditioned media. The conditioned media used were Lymfocult T (Biotest, Frankfurt, West Germany[12]) or preparations made by ourselves. They were made by similar procedures ([12]), i.e., by phytohemagglutinin (1% PHA-M) stimulation of PBMC from a pool of healthy donors. Supernatants were harvested after incubation for 48 hr (RPMI 1640 + 1% inactivated normal human serum) and stored frozen until used. The data presented are based on Lymphocult T used in a final dilution 1:200. Dilution ≥ 1:50 was found to be nonmitogenic on normal PBMC (day 3). This dilution was found to be analogous in IL-2 activity as compared to antigen-stimulated (e.g., BCG) normal PBMC. Appropriate control cultures were set up containing conditioned media but no antigen. Cultures were maintained at 37°C in 5% CO_2-humid air. For measurement of DNA synthesis, the cells were exposed to 1 μCi ^{3}H-thymidine per well on day 5 and harvested 20 hr later with a Skatron multiple cell culture harvester. ^{3}H-dThd incorporation was determined by scintillation counting (LKB). The patients (4 BL and 22 LL) were selected from the All-Africa Leprosy and Rehabilitation Training Centre (ALERT) at Addis Ababa, Ethiopia.

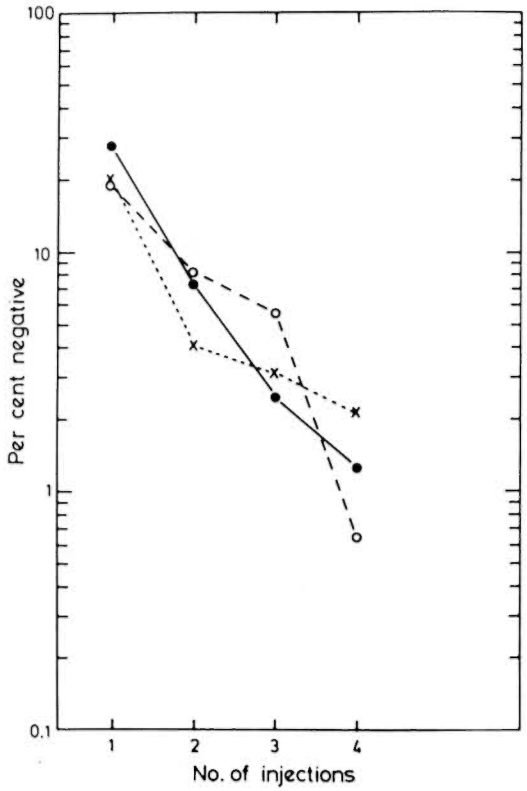

FIG. 3. Percentage of subjects remaining negative after 1–4 injections of armadillo-derived lepromin.
●——● = lepromin only
O— —O = lepromin + BCG in same site
X- - -X = lepromin + BCG in different sites

of subjects, e.g., skin test negative prior to sensitization.

The utilization of these skin tests has shown both in experimental animals and in man that lepromin, i.e., killed, intact *M. leprae*, has the capacity to induce a delayed type of skin reactivity, i.e., trigger a T cell response ([16]).

As shown by Convit and his group ([4]), the fraction that is converted to skin positivity by a standard lepromin dose is constant in a population living in a nonendemic area. By the fourth injection, about $\leq 1\%$ of the population remains skin test negative (Fig. 3).

Some major points with regard to the potency of lepromin to induce skin test conversion remain to be determined, e.g., dose. For instance, the present dose of lepromin appears to have been set to give maximal discrepancies between lepromatous and tuberculoid patients and not optimal conversion in healthy subjects. Some important studies along the line of those of Dr. Convit should be undertaken.

The study of the immunological features of repeatedly lepromin negative contacts with regard to suppressor mechanisms should be informative with regard to establishing key suppressor mechanisms involved in leprosy.

Acknowledgments. This investigation received financial support from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. AARDEN, L. A., *et al.* Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. J. Immunol. **123** (1979) 2928–2929.
2. ALVAREZ, J. M., DE LANDAZURI, M. O., BONNARD, G. D. and HERBERMAN, R. B. Cytotoxic activities of normal cultured human T cells. J. Immunol. **121** (1978) 1270–1275.
3. BLOOM, B. R. and MEHRA, V. The pathogenesis of lepromatous leprosy. In: *Immunological Aspects of Leprosy, Tuberculosis and Leishmaniasis.* D. P. Humber, ed. Amsterdam: Excerpta Medica, 1981, p. 574.
4. CONVIT, J. Report of the Fourth Meeting of the Scientific Working Group on the Immunology of Leprosy. Geneva: WHO, 1978.
5. GERMAIN, R. N. and BENACERRAF, B. A single major pathway of T lymphocyte interactions in antigen-specific immune suppression. Scand. J. Immunol. **13** (1981) 1–10.
6. GILLIS, S. and SMITH, K. A. Long-term culture of tumour-specific cytotoxic T cells. Nature **268** (1977) 154–156.
7. GLASEBROOK, A. L., SARMIENTO, M., LOKEN, M. R., DIALYNAS, D. P., QUINTANS, J., EISENBERG, L., LUTZ, C. T., WILDE, D. and FITCH, F. W. Murine T lymphocyte clones with distinct immunological functions. Immunol. Rev. **54** (1981) 255–266.
8. GODAL, T. Immunological aspects of leprosy—present status. Prog. Allergy **25** (1978) 211–242.
9. HAREGEWOIN, A., GODAL, T., MUSTAFA, A. S., BELEHU, A. and YEMANEBERHAN, T. T-cell conditioned media reverse T-cell unresponsiveness in lepromatous leprosy (submitted for publication).
10. HIETER, P. A., KORSMEYER, S. J., WALDMANN, T. A. and LEDER, P. Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. Nature **290** (1981) 368–372.

11. KURNICK, J. T., HAYWARD, A. R. and ALTEVOGT, P. Helper and suppressor-inducer activity of human T cells and their cloned progeny maintained in long-term culture. J. Immunol. **126** (1981) 1307–1311.
12. LINDSAY, P., SCHWULERA, U. and SONNEBORN, H. H. The species specificity of Interleukin 2. In: *Human Lymphokines: Biological Immune Response Modifiers.* Khan, A. and Hill, N. O., eds. New York: Academic Press, Inc., 1982, pp. 1–6.
13. MACKANESS, G. B. Delayed hypersensitivity and the mechanism of cellular resistance to infection. Prog. Immunol. **15** (1971) 413–424.
14. MACKANESS, G. B. Immunity to intracellular parasites—studies *in vivo.* Ethiop. Med. J. **11** (1973) 175–178.
15. WALKER, L. and LOWRIE, D. B. Killing of *Mycobacterium microti* by immunologically activated macrophages. Nature **293** (1981) 69–70.
16. Warren, K., ed. *Reviews of Infectious Diseases and Great Neglected Diseases: Strategies for Control.* Chicago: The University of Chicago Press, 1982.

INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Progress in the Preparation of an Antileprosy Vaccine from Armadillo-derived *Mycobacterium leprae*[1]

Richard J. W. Rees[2]

From its inception in 1974, one of the major and long-standing strategic aims of the IMMLEP program (Immunology of Leprosy), as part of the Special Programme for Research and Training in Tropical Diseases, was to explore the feasibility of developing a vaccine against leprosy ([5]). There was then, and continues now, an urgent need for procedures other than those based on chemotherapy for the control of leprosy because of the insignificant impact during the past 20 years of mass dapsone therapy on the control of leprosy, and the more recent and serious problem of dapsone resistance. A vaccine, therefore, would provide an alternative for controlling leprosy by preventing or diminishing the risk of the population at large developing the disease. Furthermore, IMMLEP was essentially encouraged to develop a specific vaccine from the then recent discovery that for the first time substantial quantities of *Mycobacterium leprae* were potentially available from experimentally infected nine-banded armadillos ([2]). Thus, two main approaches to the development of a vaccine were pursued initially: 1) to produce a vaccine based on purified, killed *M. leprae*, or 2) to identify a naturally occurring and cultivable strain of *Mycobacterium*, that had a close immunological resemblance to *M. leprae*, as a killed or live vaccine depending on its pathogenicity. Since the second approach was also dependent upon quantities of *M. leprae* only now available from infected armadillos for adequate screening of cross-reactivity with readily available and cultivable strains of mycobacteria, maximum effort and financial support was given by IMMLEP to build up and maintain adequate numbers of *M. leprae*-infected armadillos and to develop techniques for extracting and purifying *M. leprae* from the tissues of these infected animals.

In line with these priorities and the goal-oriented principles of the special program, IMMLEP contracted with several centers in the United States and one in the United Kingdom for supplying *M. leprae*-infected armadillos, with our laboratory at the National Institute for Medical Research acting as the IMMLEP *M. leprae* bank for all of these *M. leprae*-infected tissues, and with Dr. Philip Draper of our laboratory developing methods for extraction and purification of *M. leprae* from these tissues. Likewise, this goal-oriented program brought together on an international collaborative basis expertise from: Professor Morten Harboe of Oslo, Norway, in the identification of *M. leprae* antigens; Professor A. A. Juscenko of Astrakhan, USSR, in assessing purification of *M. leprae* by electronmicroscopy; Professor Barry Bloom of New York City, U.S.A., in assessing the antigenicity and immunogenicity of *M. leprae* in guinea pigs; and Dr. C. C. Shepard of Atlanta, Georgia, U.S.A., in assessing the immunogenicity of *M. leprae* as a protective vaccine, using the mouse foot pad infection.

Although equal priority was given in the first two years of the IMMLEP program for identifying strains of cultivable mycobacteria showing crossreactivity with *M. leprae*, since none were found this approach was discontinued.

There follows a summary of standardized protocols evolved by IMMLEP for establishing *M. leprae*-infected colonies of armadillos and for the extraction and purifi-

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] R. J. W. Rees, F.R.C. Path., Head, Laboratory for Leprosy and Mycobacterial Research, London NW7 1AA, England.

cation of bacteria from armadillo tissues, culminating in a biologically acceptable preparation of *M. leprae* for human studies.

Supply of *M. leprae* for IMMLEP program. Although by 1974 it had been fully established that armadillos were potentially highly susceptible to *M. leprae*, it had also been established that only some 50% of armadillos caught in the wild were susceptible. Moreover, since nine-banded armadillos had not been bred in captivity there was no way of selecting *M. leprae*-susceptible animals or of breeding mycobacterial-free colonies of armadillos. Furthermore, by 1975 there were reports of armadillos caught in the wild from parts of Louisiana, U.S.A., with mycobacterial infections indistinguishable from those produced experimentally with *M. leprae*. Therefore, from the outset IMMLEP's protocols for the supply of *M. leprae* from armadillos included rigorous criteria and examinations to exclude, as far as possible, animals with acid-fast bacillary infections, including quarantining of animals for four months and selecting animals from areas in Louisiana and Florida, U.S.A., where no *M. leprae*-like infections had been reported. To ensure maximum systemic infection in the shortest time, the animals were inoculated intravenously with not less than 1.0×10^8 *M. leprae*.

Being dependent entirely upon an *in vivo* supply of *M. leprae* from armadillos infected with bacteria from lepromatous patients, it was impossible to establish a "seeded" IMMLEP strain of *M. leprae*. To exclude the possibility of variants arising in strains of *M. leprae* serially passaged in armadillos, IMMLEP specified that all *M. leprae*-infected armadillos must be inoculated with strains of *M. leprae* from patients or from only first-passaged, armadillo-derived bacteria. However, for practical reasons the human-derived *M. leprae* were obtained from patients in widely different areas of the world and, therefore, if there are geographically determined strain differences with important antigenic or immunogenic implications, these are likely to be represented in the IMMLEP bank. The details covering all of these aspects were set out in Protocols 1/75 and 1/77 ([6, 7]).

Purification of *M. leprae* from armadillo tissues. Assuming that the IMMLEP program could provide and sustain an adequate supply of *M. leprae*-infected armadillo tissues for an eventual vaccine study, the next essential requirement and priority was to develop methods for the extraction and purification of bacteria from these tissues, based on three criteria: 1) maximum bacterial yields, 2) minimum armadillo-tissue contaminants, and 3) retention of antigenic integrity of the bacteria.

Although there were no methods described in the literature to fulfill all these criteria, Draper ([1]) had already developed methods for extracting and purifying *M. lepraemurium* from mouse tissues. IMMLEP chose to apply these methods, with Draper's assistance, to *M. leprae*-infected armadillo tissues. The first priority was to modify the *M. lepraemurium* extraction method for maximum yield of *M. leprae* from armadillo tissues. This objective was rapidly achieved by superimposing digestion of the armadillo tissues, followed by separation of any residual host tissue on sucrose gradients as used for *M. lepraemurium* (Protocol 2) ([5]). Pursuing the objective of maximum bacterial yield with the exclusion of armadillo host tissue contamination, a series of modifications were made using additional enzymatic treatment, including collagenase, trypsin/chymotrypsin, and pronase. Furthermore, a much more precise method was developed for separating tissue debris from bacteria than the sucrose gradient by applying an aqueous two-phase polymer system made up of polyethylene glycol, 6000 (PEG) and dextran, T500, referred to as Protocol 2/75 ([6]). These techniques yielded well over 95% of bacterial recovery and only minimal evidence of contamination by armadillo tissue protein, as detected by delayed-type hypersensitivity to such protein in guinea pigs sensitized to armadillo protein ([6]). However, in principle there was a serious possibility that exposure to proteolytic enzymes might well have damaged *M. leprae* sufficiently to have extracted from them some antigenic constituents, and certainly strip all operative antigens from their surface. In fact, there was evidence that purified *M. leprae* by Protocol 2/75 was less antigenic and possibly less immunogenic than cruder preparations and, in particular, a surface antigen of *M. leprae*, which at that time had been considered to be specific for *M. leprae*, had been lost from

THE TABLE. *Preparation of purified* M. leprae *from armadillo tissues.*

Object	Process
1. Effective disintegration of tissue.	1. Homogenization mechanically at high pH.
2. Inhibition of host-derived lytic enzymes—especially proteases and nucleases.	2. Homogenization and washing at high pH.
3. Control of physical form of DNA.	3. Inhibition of DNAase, presence of Mg^{++}, isotonic conditions to preserve nuclei, then hypotonic lysis in presence of DNAase.
4. Removal of bulk of insoluble tissue residues.	4. Percoll gradients (nontoxic colloidal silica).
5. Removal of traces of tissue residue with same density as bacteria.	5. Aqueous two-phase system; polyethylene glycol, 6000 + dextran, T500.
6. Removal of materials used in process.	6. Extensive washing.
7. Prevention of "clumping" of bacteria.	7. Use of 0.1% Tween 80, lightly buffered to prevent acidification due to hydrolysis of Tween 80.

bacteria extracted and purified from infected armadillo tissues by Protocol 2/75.

Priority of effort for the purification of *M. leprae* was therefore switched to the development of more gentle methods of extraction, hopefully without the use of proteolytic enzymes, but still giving high yields of bacteria free from armadillo protein. Although very many different two-phase polymer systems were tried out, none left the bacteria completely free from armadillo tissues. However, these tissue contaminants were removed in density gradients of Percoll (a stabilized and nontoxic colloidal silica) and by then partitioning the bacterial suspension in the previously developed two-phase system. Having achieved extraction without the use of proteolytic enzymes, further refinements were made at the stage of homogenization to protect the bacteria, even against possible damage from host-derived lytic enzymes, by homogenizing at pH 10 to inhibit these lysosomal enzymes. The basis of all these changes is set out in The Table, which resulted in Protocol 1/79 ([9]).

Extensive tests were undertaken for scrutinizing the purity and safety of this preparation. Electronmicroscopy revealed little or no contamination by host tissues but some electron-dense particles of Percoll. To determine the amount of Percoll contamination, a ^{125}I-labelled Percoll was prepared for use in Protocol 1/79. Only very low levels of Percoll were present, e.g., a dose of 1.6×10^7 purified *M. leprae* contained only 0.194 μg Percoll. Furthermore colloidal silica was very stable; no free crystals of silica have been detected after 25 years of storage at room temperature, and it was found not to be toxic in animals. Although there was slightly more armadillo protein contamination of bacilli prepared by Protocol 1/79 than in the protocols using proteolytic enzymes, as measured in sensitized guinea pigs, autoclaving was shown to destroy the sensitizing capacity of armadillo protein in guinea pigs. Since only autoclaved, purified *M. leprae* is envisaged for use in man, there should be no risk of human sensitization. Extensive *in vitro* and *in vivo* testing of *M. leprae* prepared by Protocol 1/79 showed no loss of identifiable mycobacterial antigens and gave good delayed-type hypersensitivity in mice and guinea pigs and optimum protective immunity in mice against challenge with live *M. leprae,* superior to that obtained with earlier preparations of purified *M. leprae* ([3]).

For a killed *M. leprae* vaccine, it was decided from the outset to kill the bacteria by inactivating the infected tissues with gamma radiation from a ^{60}Co source at a dose of 2.5 mega rad. Using the mouse foot pad test for measuring viability of *M. leprae,* it was shown that the LD50 is about 10 K rad, which for a dose of 2.5 megarad would leave only one live bacterium in 10^{61} g of bacteria.

An incidental credit from these studies has been the use of soluble blue instead of the conventional methylene blue as a more reliable and sensitive stain for detecting host-tissue contaminants ([4]).

Present position. On the basis of all of these studies, IMMLEP adopted Protocol 1/79 as the standard procedure for preparing purified *M. leprae* for their first small-scale and pre-vaccine studies in man. The

first batch for human use was prepared in licensed premises by Wellcome Research Laboratories, Beckenham, England, under contract to IMMLEP in July 1982. This batch of 9.1×10^{12} *M. leprae*, kept at −80°C, will be more than adequate for the series of vaccine studies designed to establish the safety and optimal doses of *M. leprae* for sensitization in man.

Phase I of this series is designed to establish the optimum dose of killed *M. leprae* which will sensitize but not cause unacceptable ulcers at the site of vaccination or side reactions. These tests will be undertaken on volunteers in three nonendemic areas—Norway, the United Kingdom, and the United States ([10, 11]). The tests in Norway will begin in February 1983, and those in the United Kingdom and the United States later in the year.

REFERENCES

1. DRAPER, P. The walls of *Mycobacterium lepraemurium*: Chemistry and ultrastructure. J. Gen. Microbiol. **69** (1971) 313–324.
2. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo (*Dasypus novemcinctus*, Linn.) as a model for the study of leprosy. 1. Report of lepromatoid leprosy in an experimentally infected armadillo. Int. J. Lepr. **39** (1971) 693–702.
3. SHEPARD, C. C., DRAPER, P., REES, R. J. W. and LOWE, C. Effect of purification steps on the immunogenicity of *Mycobacterium leprae*. Br. J. Exper. Pathol. **61** (1980) 375–379.
4. WHEELER, P. R. and DRAPER, P. Soluble blue as a counterstain in the Ziehl-Neelsen procedure. Int. J. Lepr. **48** (1980) 15–17.
5. WORLD HEALTH ORGANIZATION. Report of the First Meeting of IMMLEP Project Group. TDR/SWG/IMMLEP/IMM/74.3, 1974.
6. WORLD HEALTH ORGANIZATION. Report of the Second IMMLEP Task Force Meeting. Protocol 1/75. TDR/IMMLEP/75.8, 1975, p. 10.
7. WORLD HEALTH ORGANIZATION. Report of the Third IMMLEP Scientific Working Group Meeting. Protocol 1/77. TDR/SWG/IMMLEP (3)77.3, 1977.
8. WORLD HEALTH ORGANIZATION. Report of the Third IMMLEP Scientific Working Group. Protocol 3/77. TDR/SWG/IMMLEP (3)/77.3, 1977, p. 20.
9. WORLD HEALTH ORGANIZATION. Report of the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy. Protocol 1/79. TDR/SWG/IMMLEP (5) 80.3, 1980.
10. WORLD HEALTH ORGANIZATION. Protocol on vaccine studies in man to establish optimal doses for sensitisation. TDR/IMMLEP/TEST/PROTOCOL 81.1A.
11. WORLD HEALTH ORGANIZATION. Testing of purified armadillo-derived *M. leprae* in man. TDR/IMMLEP/SC/TEST/81.1.

INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Animal Vaccination Studies with *Mycobacterium leprae*[1]

Charles C. Shepard[2]

Vaccines have been studied in experimental animals since the early days of microbiology on the basis of general phenotypic resemblances. The recent flood of knowledge about the elements of the immune response system emphasizes the rationale of this approach. The immunological system in its present form appeared early in the evolution of mammals, and it has been conserved throughout the class Mammalia. Thus the immune response in mice or guinea pigs or armadillos or man to a complex (multideterminate) injected non-living antigen is apt to be very similar. In the present case our goal is maximum protection against infection, and we assume that it is achieved by attaining maximum delayed-type hypersensitivity (DTH) against *Mycobacterium leprae* antigens.

Experimental systems used for studying *M. leprae* vaccines

The experimental systems are shown in The Table. First, of course, is the injection of the vaccine. In all of the systems shown, the vaccine response is fairly slow and requires about four weeks for reasonably full development. Then, for most of the systems an eliciting antigen is injected. Often in immunology, skin tests to detect DTH make use of soluble antigens. In the case of *M. leprae*, however, comparisons have shown the intact organism to be at least as effective as the protoplasmic fraction ([1, 10]) or the cell-wall fraction ([10]). The reactions to these eliciting antigens are read 24–72 hr after injection. Some of the systems do not make use of an eliciting injection but, instead, rely on an immune reaction against the antigen in the vaccine itself (C, F, and part of D in The Table). *M. leprae* is a persisting antigen, and in a previously unimmunized animal, those local or regional lymph node reactions to the vaccine antigen develop to near completion in about 28 days. The leprologist will notice the analogy to the Fernandez and Mitsuda tests. It will be easier to comment on this point after the results with the *M. leprae*-tolerant mice are presented.

Results obtained with experimental systems

The results obtained have given clarification on a number of points of relevance to vaccination of man against leprosy.

Intact *M. leprae* have been required for a vaccine response. Physical disruption of the organism caused loss of all dectable immunogenicity ([6]). The basis for this loss is not known. There are many possible explanations, which include loss of *M. leprae's* adjuvanticity, loss or masking of relevant antigenic determinants, loss of hydrophobic packaging of the antigens, reduction in size of the antigenic package, and physical separation of adjuvanticity from immunogenicity.

Live *M. leprae* have not been required for an immune response, and heat-killed, even autoclaved, *M. leprae* are immunogenic ([1, 9]). If anything, there seems to be an increase in immunogenicity on heating. In contrast, the immunogenicity of BCG, even for *M. leprae* antigen ([9]), is decreased markedly when BCG is killed by heat. At IMMLEP's beginning, it was assumed that the immunogenicity of *M. leprae* would also decrease markedly when the organism was heat killed, and so schemes for mixing non-living *M. leprae* with an adjuvant, perhaps BCG, were considered. Because it was not necessary to proceed with the development of these schemes, significant time was saved.

[1] Received for publication on 22 November 1983; accepted for publication in revised form on 15 June 1983.

[2] C. C. Shepard, M. D., Leprosy Laboratory, General Epidemiology Branch, Bacterial Diseases Division, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, United States Department of Health and Human Services, Atlanta, Georgia 30333, U.S.A.

THE TABLE. *Experimental systems used for studying* M. leprae *vaccines.*[a]

	A. Mouse, protection against infection	B. Mouse, foot pad enlargement	C. Mouse, reaction at vaccine site and in regional lymph node	D. Mouse, other	E. Guinea pig	F. Armadillo
Vaccine						
Description	Intact Ml, usually heat-killed, usually irradiated. Aqueous.	Same as A.	Same as A.	Intact Ml, irradiated. Aqueous.	Intact Ml, irradiated. Aqueous.	Intact Ml, irradiated and heat-killed. Aqueous.
Dosage and schedule	1×10^7 Ml, once. MED 6×10^5 Ml.	1×10^7 Ml, once. MED 6×10^5 Ml.	1×10^7 Ml, once. MED 6×10^5 Ml.	100 μg (3×10^8 Ml) once. MED ~16 μg (5×10^7).	0.5 mg (1.5×10^9 Ml) once. MED 55 μg (1.6×10^8 Ml).	3×10^8 Ml.
Route and volume	ID best (flank), 0.01 ml.	Same as A.	Same as A.	Foot pad, 0.04 ml.	ID (flank), 5×0.1 ml.	ID (ear pinna), 0.10 ml.
Time to challenge	+28 days, but can be −60 to ≥+200 days.	+28 days, but can be ≥+200 days.	No challenge.	Usually 28 days.	1 mo, but can be 365 days.	No challenge.
Challenge						
Description	Mouse passage Ml.	Intact Ml.	No challenge.	Various.	Intact Ml, Ml soluble antigen, PPD.	No challenge.
Dosage	5×10^3 Ml, foot pad.	1×10^7 Ml, foot pad.	No challenge.	—	6 μg of each (≃2×10^7 for intact Ml) ID.	No challenge.
Measurement and time	After controls reach plateau (ca. 6 mo), count AFB in 8 individual mice. Compare with counts from controls. Repeat in 90 days.	Measure FPE at 24, 48, 72 hr. Need 5–10 mice per group.	Measure swelling at vaccine site and in regional lymph node at 28 days.	Swelling at vaccine site. FPE to PPD. FPE to Ml-soluble antigen. Resistance to *M. tuberculosis* (R1Rv) challenge. Local resistance to *Listerella monocytogenes* challenge.	Induration at 24 hr.	Measure induration at vaccine site at 28 days.
References	4–10	10	5–10	2	1	C. C. Shepard, unpublished results.

[a] Abbreviations: Ml = *M. leprae*; MED = minimal effective dose; ID = intradermally; PPD = purified protein derivative from *M. tuberculosis*; AFB = acid-fast bacteria; FPE = foot pad enlargement.

Methods for purifying *M. leprae* from armadillo tissue. Dr. Rees has just discussed the methods of purification ([3]). Results in mice suggest that treatment with protective enzymes is detrimental to the immunogenicity of *M. leprae* suspensions ([5, 6]). The other technical manipulations (centrifugal washing, separation on Percoll gradients, and separation in DEAE-dextran : polyethylene glycol two-phase systems) are not harmful.

Of the cultivable mycobacteria, only BCG has given solid and consistent protection against *M. leprae*. Among the various IMMLEP approaches to the development of a vaccine was a search among cultivable mycobacteria for better protection against *M. leprae*, but the search has only emphasized the position of BCG. Even H37Ra, an avirulent human tubercle bacillus, has been ineffective. Most of these studies have been carried out in mice by means of foot pad enlargement (FPE) tests with *M. leprae* as the challenge antigen and infectious-challenge tests with *M. leprae* ([9]). Some of the cultures have given no evidence of being immunogenic for themselves, but others have and they also have shown no significant crossreactivity with *M. leprae*.

Minimal effective vaccine dose for *M. leprae* and BCG. Because of the possibility that differences between *M. leprae* and BCG were being obscured by flat dose-response curves, we have carried out an unpublished titration of heat-killed *M. leprae*, live BCG, and a mixture of heat-killed *M. leprae* and live BCG in mice. The doses of acid-fast bacteria (AFB) tested were (in terms of $\log_{10}$) 7.6, 7.0, 6.4, 5.8, and 5.2. The measurements consisted of FPE with *M. leprae* challenge, reaction at the vaccine site, and enlargement of regional lymph nodes at 28 days, and protection against foot pad infection with *M. leprae*. The essential finding was that FPE and protection was about the same for all three vaccines with the middle doses; FPE and protection were somewhat less at the smallest dose with *M. leprae*, and somewhat less at the largest dose with BCG. The reactions at the vaccine site and the enlargement of the regional lymph nodes (what we would consider side-effects in humans) tended to be greater with BCG and the mixture than with *M. leprae* alone at each dose. Thus the results emphasize the near equivalence of these three products in previously unimmunized animals and suggest that the choice among them for human vaccine will need to be based on comparisons in man.

Repetition of vaccination. In guinea pigs, little was gained by repeating the vaccination. The reason seems to be that *M. leprae* antigens persist very well in the tissues after a single dose.

Duration of immune response. In mice, vaccine protection against infection with *M. leprae* lasts at least a year after vaccination with BCG ([10]); vaccine protection and DTH (measured by FPE) lasts at least a year after vaccination with heat-killed *M. leprae* (unpublished observations). In guinea pigs, DTH lasts at least one year ([1]). Since regional lymph node enlargement seems to last for the life of the mouse, DTH and protection probably last as long also. The persisting lymph node enlargement signifies persisting *M. leprae* antigen, either at the intradermal site of injection or in the regional lymph node. One hopes that the DTH in man will last longer than grossly observable reactions at the vaccine site and regional lymph nodes.

Correlation between DTH and protection against infection. In mice, DTH, as measured by FPE after injection of intact, heat-killed *M. leprae*, has correlated reasonably well with protection against infection. The minor differences that have been observed appear to be related to differences in the timing of the manifestation of immunity for the two measurements and to differences in the dose-response curve.

Genetic basis for differences in immune response. The response in the lines of mice and guinea pigs that have been tested has been uniform, but more lines need to be studied.

Comparison of the minimal intradermal immunogenic dose in animal species. The minimal effective dose (MED) of heat-killed, Percoll-purified *M. leprae* in mice was found to be about 6×10^5 bacilli in one titration. Mehra and Bloom ([1]) found the MED in guinea pigs to be 1.6×10^8 bacilli; their bacilli, however, were purified by the early enzyme method. In armadillos we find that a dose of 3×10^8 bacilli will give a fairly consistent, local 28-day reaction. In man the MED, as determined by Mitsuda reac-

tions, seems to be about 1.6×10^7 bacilli. These determinations, however, lump together previously unsensitized persons with persons having pre-existing immunity. The MED for positive Mitsuda reactions might be higher in persons with negative Fernandez tests than in those with positive tests.

Route of immunization and tolerance ([11]). (The term tolerance is used here noncommittally as regards mechanism.) Recently we have observed that the intradermal route is much more immunogenic for mice than other routes tried. On the other hand, tolerance could be induced efficiently by intravenous injections of *M. leprae* suspensions. Tolerance was measured by intradermal challenge with *M. leprae* followed in 28 days by tests of FPE after foot pad challenge with *M. leprae*. The tolerance was long lasting (at least 112 days). It could be partially overcome by intradermal injections of living BCG and, to an equal extent, by mixtures of BCG and *M. leprae*. The immune response to the intradermal injections of *M. leprae* could be prevented by prior treatment with ultraviolet light (UV-B). The minimal effective dose appears to be low and within the range provided by exposure to sunlight under normal circumstances. Histological studies show that intradermally injected carbon particles deposit in the dermis, with a maximum depth of about 0.3 mm or about the same as the maximum depth of penetration of 95% of the UV-B.

The 28-day reactions at the site of the intradermal *M. leprae* injection and in the regional lymph nodes conformed to expectations if these phenomena depend upon the formation of DTH, that is, the reactions were negative in the tolerant mice and positive in the normal mice. The reactions at the vaccine site are of course analogous to the Mitsuda reactions. By this analogy the Mitsuda reaction can be viewed as a test for immune tolerance. Thus a negative Mitsuda reaction would signify the presence of tolerance. A positive reaction would signify the absence of tolerance, and the presence of an immune response to *M. leprae*. The immune response could be the result of preexisting immunity or of successful primary vaccination with the Mitsuda antigen itself. In this consideration, there is an implication that tolerance is an active immunological process, even though the mechanism in mice (e.g., suppressor cells) has not been worked out.

In conclusion, let me restate the goals of animal vaccination studies with *M. leprae*. They lie in two general areas. One has to do with the preparation of antigen for the vaccine. The solution we have arrived at for *M. leprae* is fairly simple. One prepares purified suspensions of *M. leprae* from infected armadillo tissues and autoclaves them. This product is nicely immunogenic in animals and, based on its similarity to integral lepromin, will probably be immunogenic in man. The other general area involves studies of the ways in which particular elements of the immune system can be most advantageously engaged in order to produce a strong and long-lasting DTH to *M. leprae*, even in individuals who have immune tolerance to *M. leprae*. In this area progress has been made, but more studies are needed.

REFERENCES

1. Mehra, V. and Bloom, B. R. Induction of cell-mediated immunity to *Mycobacterium leprae* in guinea pigs. Infect. Immun. **23** (1979) 787–794.
2. Patel, P. J. and Lefford, M. J. Induction of cell-mediated immunity to *Mycobacterium leprae* in mice. Infect. Immun. **19** (1979) 87–93.
3. Rees, R. J. W. Progress in the preparation of an antileprosy vaccine from armadillo-derived *Mycobacterium leprae*. Int. J. Lepr. **51** (1983) 515–518.
4. Shepard, C. C. Vaccination of mice against *M. leprae* infection. Int. J. Lepr. **44** (1976) 222–226.
5. Shepard, C. C., Draper, P., Rees, R. J. W. and Lowe, C. Effect of purification steps on the immunogenicity of *Mycobacterium leprae*. Br. J. Exp. Pathol. **61** (1980) 376–379.
6. Shepard, C. C., Minagawa, F., Van Landingham, R. and Walker, L. L. Foot pad enlargement as a measure of induced immunity to *Mycobacterium leprae*. Int. J. Lepr. **48** (1980) 371–381.
7. Shepard, C. C., Van Landingham, R. and Walker, L. L. Immunity to *Mycobacterium leprae* infections stimulated by *M. leprae*, BCG, and graft-versus-host reactions. Infect. Immun. **14** (1976) 919–928.
8. Shepard, C. C., Van Landingham, R. and Walker, L. L. Searches among mycobacterial cultures for antileprosy vaccines. Infect. Immun. **29** (1980) 1034–1039.
9. Shepard, C. C., Walker, L. L. and Van Landingham, R. Heat stability of *Mycobacterium leprae* immunogenicity. Infect. Immun. **22** (1978) 87–93.

10. SHEPARD, C. C., WALKER, L. L. and VAN LANDINGHAM, R. M. Immunity to *Mycobacterium leprae* infections induced in mice by BCG vaccination at different times before or after challenge. Infect. Immun. **19** (1978) 391–394.

11. SHEPARD, C. C., WALKER, L. L., VAN LANDINGHAM, R. M. and YE, S-Z. Sensitization or tolerance to *Mycobacterium leprae* antigen according to its route of injection (manuscript in preparation).

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Serodiagnosis of Infection with *Mycobacterium leprae*[1]

Thomas M. Buchanan, Douglas B. Young,
Richard A. Miller, and Saroj R. Khanolkar[2]

This review is confined to serodiagnostic methods reported to be specific for infection with the leprosy bacillus, or to methods of potential serodiagnostic value that utilize chemically defined and pure antigens of *Mycobacterium leprae*. These methods are the indirect fluorescent antibody test (FLA-ABS) described by Abe, *et al.* (1); the radioimmunoassay (RIA) procedure developed by Harboe, *et al.* (6); agar gel immunodiffusion precipitation techniques reported by Caldwell, *et al.* (2) or Payne, *et al.* (9); an ELISA procedure utilizing purified arabinomannan described by Miller, *et al.* (8); and ELISA procedures employing purified phenolic glycolipid of *M. leprae* either in its native form (Hunter, *et al.* (7)) or as the deacylated molecule (Young and Buchanan (10)).

Experimental procedures and results

The RIA of Harboe, *et al.* (6); the agar gel diffusion immunoprecipitation procedure of Caldwell, *et al.* (2); and the FLA-ABS test of Abe (1) each utilize crude antigen extracts or whole *M. leprae*. In contrast, the agarose gel immunodiffusion precipitation procedure of Payne, *et al.* (9) employs liposomes containing purified native phenolic glycolipid and the ELISA methods of Miller, *et al.* (8); Hunter, *et al.* (7); or Young and Buchanan (10) employ purified molecules: arabinomannan, native phenolic glycolipid and deacylated phenolic glycolipid, respectively (Table 1). The RIA described by Harboe, *et al.* (6) and the FLA-ABS of Abe, *et al.* (1) each require preadsorption of all sera tested in order to attain specificity. Sonicated BCG is used for adsorption in the RIA, and BCG, *M. vaccae*, cardiolipin and lecithin are used for adsorption in the FLA-ABS (Table 1). In addition, in the FLA-ABS the fluorescein conjugate is preadsorbed with a 2.5% (w/v) final concentration of a sonicate of BCG, and each serum tested is diluted beyond 1:10 in phosphate-buffered saline (PBS) containing BCG sonicate and bovine serum albumin (BSA). Adsorption of only the reference serum is required in the procedure described by Caldwell, *et al.* (2) and no adsorptions are needed for the procedure of Payne, *et al.* or the ELISA tests employing purified molecules (Table 1). IgG is the primary immunoglobulin class detected in four of the tests: 1) the RIA procedure that employs protein A (6), 2) the ELISA procedure using arabinomannan and a gamma chain specific enzyme conjugate (8), 3) the FLA-ABS test using whole organisms, and 4) the ELISA employing native phenolic glycolipid. Both 3 and 4 employ enzyme conjugates linked to antibodies directed at human IgG.

The only ELISA assay that readily detects IgM antibody is the technique described by Young and Buchanan that employs deacylated phenolic glycolipid (PG) and an enzyme conjugate linked to antibodies to human IgM, IgG, and IgA (Table 1). This may be important since the dominant human immune response to the phenolic glycolipid specific antigen of *M. leprae* appears to be IgM (Table 2). The relative inability of both the FLA-ABS test and the ELISA procedure described by Hunter, *et al.* to recognize IgM

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] T. M. Buchanan, M. D., Departments of Medicine and Pathobiology, University of Washington; Immunology Research Laboratory, Seattle Public Health Hospital, Seattle, Washington, and National Hansen's Disease Center, Carville, Louisiana 70721. D. B. Young, Ph.D., and S. R. Khanolkar, Ph.D., Department of Pathobiology, University of Washington and Immunology Research Laboratory, Seattle Public Health Hospital, Seattle, Washington. R. A. Miller, M.D., Department of Medicine, University of Washington and Immunology Research Laboratory, Seattle Public Health Hospital, Seattle, Washington, U.S.A.

TABLE 1. *Serologic tests to detect antibody to* M. leprae *antigens.*

Author (reference)	Test	Antigen preparation	Antigen recognized	Preadsorption of sera	Immunoglobulin class detected
Harboe, *et al.* ([6]), 1978	RIA	*M. leprae* sonicate 15 min @ 80 watts supt. 20,000 × *g*	Protein CIE Ag 4 <300,000 m.w. ^{125}I-labeled	BCG 60 mg sonicated 15 min 80 watt, 20,000 × *g* supt. of sonicate used in 1:2 dilution with serum.	IgG recognized by protein A
Caldwell, *et al.* ([2]), 1979	Immuno-diffusion	Lithium acetate alkaline extract of *M. leprae*	Protein, destroyed by heat or proteases	Reference serum only, adsorbed in 1:10 dilution and final concentrations of 0.167% BCG (w/v), 0.33% *M. vaccae*, 0.02% cardiolipin, 0.02% lecithin. Antibodies in test serum recognized by line of identity with precipitins formed by reference serum.	IgG, IgM, IgA
Abe, *et al.* ([1]), 1980	FLA-ABS	Whole *M. leprae* 10 min CCl_4, 20°C; 60 min 0.1% trypsin, 37°C	Surface antigen	2nd Ab-FA conjugate: 2.5% BCG sonicate, 30 min, 37°C. Each test serum; 1:10 dilution in PBS with final concentrations of the following compounds in 0.167% (w/v) BCG, 0.33% (v/v) *M. vaccae*, 0.02% cardiolipin, 0.02% lecithin.	IgG ≫ IgM, IgA (anti-IgG)
Payne, *et al.* ([9]), 1982	Immuno-diffusion	Purified native phenolic glycolipid incorporated into liposomes	Native phenolic glycolipid	None required	IgG, IgM, IgA
Hunter, *et al.* ([7]), 1982	ELISA	Purified native phenolic glycolipid	Native phenolic glycolipid	None required	IgG ≫ IgM, IgA (anti-IgG enzyme conjugate)
Miller, *et al.* ([8]), 1983	ELISA	Purified arabinomannan	Arabinomannan	None required	IgG (γ chain specific enzyme conjugate)
Young and Buchanan ([10]), submitted	ELISA	Purified deacylated phenolic glycolipid	Deacylated phenolic glycolipid	None required	IgG, IgM, IgA (anti-G, M, and A enzyme conjugate)

TABLE 2. *Human antibody levels to phenolic glycolipid measured by ELISA in serum from 24 leprosy patients.*[a]

Enzyme conjugate	No. patients with A_{492} >0.1	Mean ΔA_{429}[b] for 24 patients
Anti-IgG, IgM, IgA	24	0.89
Anti-IgM	24	0.98
Anti-IgG	14	0.22
Anti-IgA	17	0.28

[a] Includes the following Ridley-Jopling patient classifications: 1 TT, 4 BT, 4 BB, 3 BL, and 12 LL.

[b] ΔA_{492} = the absorbance at 492 nm wave length developed for a given serum in wells containing phenolic glycolipid antigen minus the absorbence developed in wells on the same polystyrene plate that contained no antigen.

TABLE 3. *Removal of phenolic glycolipid (PG) from polyvinyl plates due to washing with Tween 80.*[a]

No. washes PBS with 0.05% Tween 80[a]	Δcpm[b] ^{125}I-monoclonal antibody to PG	% Total cpm
0	8773	100
1	2463	28
2	1351	15
3	1274	14

[a] Plates were coated with native phenolic glycolipid in hexane, reacted with ^{125}I-monoclonal antibody to PG, and washed four times with phosphate buffered saline (PBS). The table shows the effect of adding Tween 80 in one or more of the washing steps as described by Hunter, *et al.* (7).

[b] Δcpm = cpm of ^{125}I-monoclonal antibody bound to antigen-coated wells minus the cpm of ^{125}I-antibody bound to wells containing no antigen.

antibodies may therefore compromise the ability of these tests to register the human immune response to phenolic glycolipid. Other procedural factors affect the performance of the ELISA tests for antibodies to phenolic glycolipid as described by Hunter, *et al.* (7) when compared to the procedure of Young and Buchanan (10). For example, the hexane utilized by Hunter, *et al.* reacts with the structural polymers in polyvinyl chloride and polystyrene microtiter plates, resulting in a four-to-tenfold increase in nonspecific binding of antibody to the hexane-treated plates. Tween 80, as utilized in the washes between steps in the ELISA procedure of Hunter, *et al.* (7) solubilizes the phenolic glycolipid antigen. As shown in Table 3, as much as 72% of the native glycolipid molecule may be removed by a single wash with PBS containing 0.05% Tween 80, one half of the Tween concentration utilized in that procedure (7). Perhaps the major difference in ELISA performance may result from the use of deacylated PG as compared to the native PG molecule. For example, we found that a concentration of 32 μg/ml of the purified native PG produced a ΔA_{492} of 0.24 as compared to a ΔA_{492} of 0.32 produced by only 1 μg/ml of the purified deacylated PG (10). Thus, once separated from its natural configuration in the cell wall of the leprosy bacillus, the purified deacylated molecule had approximately 40-fold greater antigenicity than the purified native PG when used in aqueous systems such as the ELISA. In addition, only 4 μg/ml concentrations of the deacylated molecule were required to produce ΔA_{492} of 1.2, an absorbence level not possible with practical concentrations of the purified native PG. These differences in ELISA performance of the purified native and deacylated PG molecules presumably relate to the hydrophobicity of the native molecule and its insolubility in aqueous immunoassay systems, resulting in concealment of the unique trisaccharide antigenic moiety by the excess lipid. When the molecule is deacylated, approximately one half of the total lipid of the native PG is removed, and 50 μg/ml concentrations of the deacylated molecule form a clear solution, in contrast to the visibly turbid suspension character of the same concentrations of the native PG.

The practical effects of the methodologic differences for the ELISA procedures of Hunter, *et al.* (6) and Young and Buchanan (10) were examined by testing the same sera from six normal individuals and ten patients with the two systems. Sera from normal persons could not be distinguished from the patients' sera by the described method of Hunter, *et al.*, and none of the patients had detectable elevated antibody levels, presumably for the methodological reasons noted above. In contrast, eight of the ten patients and none of the normal individuals had elevated antibody levels to phenolic

glycolipid when tested with the ELISA employing the deacylated molecule, utilizing no hexane and no Tween in the washes, and using an enzyme conjugate capable of detecting IgM antibodies.

Table 4 summarizes reported performances with serum from leprosy patients, household contacts of leprosy patients, and controls for each of the serodiagnostic tests listed in Table 1. The two procedures based upon protein antigens specific for *M. leprae* (Harboe, *et al.* [6] and Caldwell, *et al.* [2]) each required 60 mg and 100 mg dry weight of bacilli, respectively, to prepare the initial antigen mixture. The FLA-ABS test requires approximately 2×10^6 whole bacilli per test, and the methods employing chemically defined antigens require 1 μg–9 μg of purified molecules per serum specimen tested (Table 5). The procedure requiring the least amount of antigen (Young and Buchanan [10]) utilizes 0.5 μg of purified deacylated PG coated to each well of a polystyrene microtiter plate, or 1 μg per test since all specimens are tested in duplicate. The quantity of antigen required per test is the limiting factor for practical use, especially for those tests based upon the specific protein antigens of *M. leprae*. Utilization of the phenolic glycolipid antigen is advantageous since it is exposed on the surface of the leprosy bacillus, and it is synthesized in quantitites approximately 100-fold greater than the amounts of specific proteins thus far identified. The widespread use of serodiagnostic tests based upon specific protein antigens of *M. leprae* may therefore require genetic engineering or other developments to increase the availability of specific proteins of the organism. In contrast, the amount of phenolic glycolipid antigen present in an armadillo liver moderately infected with *M. leprae* is estimated to be sufficient for approximately 20,000 tests using the ELISA procedure of Young and Buchanan [10]. In addition, 90% of this glycolipid can be isolated as a by-product of the purification of bacilli from infected tissue. In the Immunology Research Laboratory of the Seattle Public Health Hospital, approximately 3000 sera have been tested with this procedure during the past six months as part of the evaluations of patient and contact populations in Mexico and Sri Lanka.

As shown in Table 4 only four of the seven methods have been tested with large panels of sera. These are the RIA procedure of Harboe, *et al.* [6]; the FLA-ABS test of Abe, *et al.* [1]; the ELISA with arabinomannan antigen of Miller, *et al.* [8]; and the ELISA employing deacylated phenolic glycolipid [10]. A total of 53 sera were tested in the immunodiffusion precipitation procedure of Caldwell, *et al.* [2], and even smaller numbers of human sera were tested in the procedures of Payne, *et al.* (8 sera [9]) and Hunter, *et al.* (3 sera [7]). All four procedures tested against large numbers of human sera showed high levels of sensitivity. In general, each procedure showed higher antibody levels in serum from the more bacilliferous patients, suggesting a direct correlation between antigen load and the amount of antibody detected. The specificity for infection with the leprosy bacillus was greater for the three procedures employing *M. leprae*-specific antigens than for the ELISA employing arabinomannan, an antigen common to most mycobacteria and *Nocardia*. The arabinomannan ELISA was positive in sera from 14 of 25 patients with pulmonary tuberculosis, in contrast to 0/8, 0/18, or 0/10 tested by the procedures of Harboe, *et al.*; Abe, *et al.*; or Young and Buchanan, respectively (Table 4). Even though the arabinomannan ELISA was nonspecific, it was hoped that its sensitivity would be great enough so as to not exclude any persons with *M. leprae* infection. This has not proven to be the case if one accepts seropositivity in the ELISA procedure employing deacylated PG as evidence of infection. The concordance between the two tests was surprisingly low when they were used to test approximately 1000 household contacts of leprosy patients in Mexico and Sri Lanka, and many contacts with elevated antibody levels to PG did not have elevated antibodies to arabinomannan.

Other control sera include BCG vaccinated individuals, persons with mycobacterial infections other than tuberculosis, and normal individuals with no known infections or BCG vaccinations. Abe, *et al.* reported that the FLA-ABS test was negative with serum specimens from 50 normal persons, and they did not test sera from persons with BCG vaccination or other mycobacterial infections [1] (Table 4). Harboe, *et al.*

TABLE 4. *Performance characteristics of different serologic tests for detection of infection with* M. leprae.

Author (reference)	Test	Antigen required	Leprosy patients (no. positive/total)					Contacts of leprosy patients	Controls			
			LL	BL	BB	BT	TT		Tbc.	Other myco.	BCG vac.	Normal
Harboe, *et al.* (6), 1978	RIA	60 mg[a]	28/28	23/24	12/12	10/24	2/8	—	0/8	—[b]	0/30	—
Caldwell, *et al.* (2), 1979	Immuno-diffusion	100 mg[a]	15/15	—	—	5/7	—	—	—	—	1/16	0/15
Abe, *et al.* (1), 1980	FLA-ABS	2×10^6	128/129	8/8	12/12	7/8	13/17	57/62	0/18	—	—	0/50
Payne, *et al.* (9), 1982	Immuno-diffusion	9 μg	3/3	—	—	0/2	0/2	—	0/2	—	—	—
Hunter, *et al.* (7), 1982	ELISA	5 μg	2/2	—	—	—	—	—	—	—	—	0/1
Miller, *et al.* (8), 1983	ELISA	4 μg	35/36	—	—	—	21/46	41/256	14/25	—	4/16	1/22
Young and Buchanan (10)	ELISA	1 μg	12/12	11/12	9/12	7/12	5/12	—[c]	0/10	0/10	0/10	0/10

[a] Harboe, *et al.* utilized 60 mg dry weight of leprosy bacilli to prepare each sonicate preparation prior to radiolabeling and use in the RIA. Caldwell, *et al.* utilized 3×10^{10} bacilli to prepare each lithium acetate extract of antigen for use in the immunodiffusion precipitation test.

[b] Harboe, *et al.* found that rabbit antiserum to *M. nonchromogenicum* or to *M. avium* produced positive results in the RIA test, even after adsorption with BCG sonicate.

[c] When the ELISA procedure of Young and Buchanan was used to test household contacts of leprosy patients, 218/597 (36%) and 189/898 (21%) were seropositive in Sri Lanka and Mexico, respectively (unpublished data).

found that the RIA was negative with serum from 30 persons who had received BCG vaccination, and they did not report on the results with sera from normal individuals or persons with other mycobacterial infections. It was found, however, that the RIA test was reactive when rabbit antisera to *M. avium* or to *M. nonchromogenicum* were used. Thus, serum from patients with *M. avium-intracellulare* and other non-tuberculous mycobacterial infections will need to be tested to further establish the specificity of the serologic detection of leprosy based upon *M. leprae* specific protein antigens. When the procedure of Caldwell, *et al.* [2] was utilized to examine extracts of 22 species of mycobacteria by Gillis, *et al.* [5], evidence was found for significant crossreactivity of protein antigens among *M. leprae, M. bovis,* and *M. lepraemurium.* Subsequent studies with monoclonal antibodies to the protein antigens of *M. leprae* suggest that a protein of 68,000 daltons subunit molecular mass may contain both shared and *M. leprae*-specific determinants (Gillis and Buchanan [5], and unpublished data). A further consideration when employing a serodiagnostic test based upon the protein antigens of *M. leprae* is that proteolysis occurs during the purification of the bacilli from infected liver tissue unless protease inhibitors such as 1 mM benzamidine are included within the buffers during the purification (Buchanan, unpublished data).

The ELISA procedure employing deacylated phenolic glycolipid found no seropositives among any of 10 BCG-vaccinated individuals, 10 persons with other mycobacterial infections including *M. avium-intracellulare,* or 10 normal individuals [10]. Results for sera from contacts of leprosy patients have not been reported for the RIA procedure of Harboe, *et al.* Abe, *et al.* reported that 57 of 62 household contacts of leprosy patients were seropositive in the FLA-ABS test [1]. The seropositivity rate in household contacts is lower for the ELISA procedure of Young and Buchanan than for the FLA-ABS test. Using the ELISA for antibodies to deacylated PG, we have found 218/597 (36%) and 189/898 (21%) of household contacts of leprosy patients that were seropositive in Sri Lanka and Mexico, respectively.

DISCUSSION

From the available data, the FLA-ABS test of Abe, *et al.* [1], and the ELISA procedure of Young and Buchanan [10], appear most promising for the serodiagnosis of infection with *M. leprae.* More studies are needed to compare these two procedures utilizing the same sera from different patient and contact populations. In a group of sera from 80 household contacts of leprosy patients in Mexico that were tested by the FLA-ABS, and by the ELISA procedures for antibodies to arabinomannan or to deacylated PG, 46 were positive with the FLA-ABS test, 16 were positive with the ELISA for antibodies to arabinomannan, and 7 had elevated antibodies to PG as measured by ELISA [3].

The usefulness of any serologic test designed to permit the early diagnosis of new leprosy cases will be dependent upon its ability to predict which individuals will subsequently develop clinical leprosy. In other words, it is not enough to be able to detect antibodies specific for the leprosy bacillus, since these antibodies may reflect an immune response to dead bacilli, or to bacilli present in sufficiently small quantities that the patient rejects the infection and no clinical disease results. For a test to be practical, it must provide criteria for the accurate prediction of which persons will subsequently develop clinical leprosy. In this regard, the ELISA procedure of Young and Buchanan might appear more promising, since seropositivity rates with this test are 21%–35% in household contacts of leprosy patients, as compared to rates of 92% for the FLA-ABS test. Since approximately 10% of household contacts of patients with lepromatous leprosy develop the disease, and an even smaller percentage of household contacts of tuberculoid patients develop leprosy, the percent of seropositives that will subsequently develop leprosy must be higher for the ELISA with deacylated PG than for the FLA-ABS test. However, it may be possible to select quantitative criteria, based upon the ΔA_{492} in the ELISA, or the titer in the FLA-ABS, that may select individuals with a greater risk of developing disease. For example, the mean ΔA_{492} in the ELISA employing deacylated PG for 66 patients with

LL or BL leprosy was 0.62, and the ΔA_{492} for 63 BT patients and 71 TT patients was 0.33 and 0.11, respectively. This may imply that a serodiagnostic test for infection with *M. leprae* may have more usefulness for early detection of lepromatous disease, since with these patients there may be a 4–6-year period before the diagnosis is made clinically, during which time antibody levels are increasing and bacilli are present. Further studies with both serologic tests using sera from the same populations should eventually determine their relative merits, and their usefulness for the early detection of leprosy.

Acknowledgments. We are grateful to James P. Harnish, M.D., Sen Dissanayake, Ph.D., Sergio Estrada-Parra, Ph.D., Fausto Quesada-Pascual, Ph.D., and J. R. Acedo, M.D., for assistance with the classification of patients and the collection of sera. This research was supported in part by the Immunology of Leprosy (IMMLEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, by the Rockefeller Foundation Program for Research in Great Neglected Diseases, by NIH grant AI16290, and by the Victor Heiser Foundation Fellowship Program. Armadillo tissue infected with *M. leprae* was obtained from NIAID, NIH, and WHO supported armadillo colonies.

REFERENCES

1. Abe, M., Minagawa, F., Yoshino, Y., Ozawa, T., Saikawa, K. and Saito, T. Fluorescent leprosy antibody absorption (FLA-ABS) test for detecting subclinical infection with *Mycobacterium leprae*. Int. J. Lepr. **48** (1980) 109–119.
2. Caldwell, H. D., Kirchheimer, W. F. and Buchanan, T. M. Identification of a *Mycobacterium leprae* specific protein antigen and its possible application for the serodiagnosis of leprosy. Int. J. Lepr. **47** (1979) 477–483.
3. Estrada-G., I., Miller, R. A., Rojas-Espinosa, O., Garcia-Ortigoza, E., Young, D. B. and Buchanan, T. M. Comparison of four serologic tests for detection of infection with *Mycobacterium leprae* using 100 sera from patients and household contacts (manuscript in preparation).
4. Gillis, T. P., Abe, M., Bullock, W. E., Rojas-Espinosa, O., Garcia-Ortigoza, E., Draper, P., Kirchheimer, W. and Buchanan, T. M. Comparison of 22 species of mycobacteria by immunodiffusion against an absorbed reference leprosy serum. Int. J. Lepr. **49** (1981) 287–293.
5. Gillis, T. P. and Buchanan, T. M. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **37** (1982) 172–178.
6. Harboe, M., Closs, O., Bjune, G., Kronvall, G. and Axelsen, N. H. *Mycobacterium leprae* specific antibodies detected by radioimmunoassay. Scand. J. Immunol. **7** (1978) 111–120.
7. Hunter, S. W., Fujiwara, T. and Brennan, P. J. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257** (1982) 15071–15078.
8. Miller, R. A., Dissanayake, S. and Buchanan, T. M. Development of an enzyme-linked immunosorbent assay using arabinomannan from *M. smegmatis:* A potentially useful screening test for the diagnosis of incubating leprosy. Am. J. Trop. Med. Hyg. **32** (1983) 555–564.
9. Payne, S. N., Draper, P. and Rees, R. J. W. Serological activity of purified glycolipid from *Mycobacterium leprae*. Int. J. Lepr. **50** (1982) 220–221.
10. Young, D. B. and Buchanan, T. M. A serological test for leprosy using a glycolipid specific for *Mycobacterium leprae* (submitted for publication).

INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Investigations Related to the Development of a Leprosy Vaccine[1]

Jacinto Convit, Nacarid Aranzazu, Marian Ulrich, Manuel Zúñiga, Maria Eugenia de Aragón, Jorge Alvarado, and Oscar Reyes[2]

The clinicopathological spectrum of leprosy reflects the immunological response of the human host to *Mycobacterium leprae*. There is considerable evidence to indicate that the immunological defect which reaches its maximum expression in lepromatous patients also occurs in other groups of individuals, including a small proportion of the healthy population in endemic areas. Many of the principles which have been applied to the immunotherapy of leprosy patients are useful in the development of an experimental design for prophylactic vaccination in leprosy. This paper will review our experience in the immunotherapy of more than 500 leprosy patients with a mixture of heat-killed *M. leprae* and live BCG. The criteria used to identify the high-risk group of healthy individuals are presented, as well as the preliminary results of the application of the *M. leprae*-BCG vaccine in this group.

The lepromatous polar form of leprosy, LL, represents the consequence of a specific immunological defect which persists throughout the lifespan of the patient. Considerable evidence suggests that both macrophages and T lymphocytes show defective responses in polar LL. The macrophage defect manifests itself in the lack of adequate presentation of specific antigens to the T cell ([3, 4, 10, 12, 16]). The lymphocyte defect is characterized by the depression of lymphocyte transformation and the absence of lymphokine production in the presence of specific antigens ([1, 8, 14, 15]). Both of these cell types are also involved in immunoregulatory processes which are altered in LL. One of the mechanisms which has been suggested to explain the defective immune response in these patients is the presence of suppressor cells ([13]).

Although the mechanism of the immunological defect in the lepromatous patient has only been partially clarified, the specificity of the defect is evident. These patients demonstrate a normal capacity to defend themselves from diverse infecto-contagious diseases and do not show defects in tumor surveillance or other evidence of immunodeficiency.

We visualize the mechanisms of the immunological defect in cell mediated immunity (CMI) in lepromatous leprosy in the following terms: a primary defect in the presentation of specific antigens to the T lymphocyte by macrophages results in a failure of the development of an adequate T cell response by the lymphoid arm of the immune system. Additionally, inappropriate antigen presentation by, or bypassing of, macrophages may stimulate the formation of suppressor cells.

The results observed after immunotherapy with a mixture of *M. leprae* and BCG in patients and persistently Mitsuda-negative contacts suggests that the CMI defect in lepromatous leprosy is not an irreversible central deficiency, but rather a peripheral defect which can be overcome by an adequate regimen of immunotherapy. *In vivo* tests have demonstrated that lepromatous patients, as well as an undetermined proportion of patients with indeterminate leprosy and persistently Mitsuda-negative contacts, are unable to eliminate heat-killed *M.*

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] J. Convit, M.D.; N. Aranzazu, M.D.; M. Ulrich, Ph.D.; M. Zúñiga, M.D.; M. E. de Aragón, B.S.; J. Alvarado, M.D.; O. Reyes, M.D., Instituto Nacional de Dermatologia, Apartado Postal 40403, Caracas 101, Venezuela.

leprae from their tissues ([3]). The intracutaneous injection of 6×10^7 heat-killed *M. leprae* in LL patients results in the formation of an incompetent macrophage granuloma; the injection of a mixture of *M. leprae* and BCG results in an immune granuloma with elimination of both mycobacteria ([4]). This visual evidence of an enhancement of macrophage digestion at the injection site does not prove by itself the generation of specific immunogens of *M. leprae*. However, considerable clinical, histopathological, and immunological evidence has been accumulated over more than a decade of investigation which demonstrates that a variable number of injections indeed produces responses to antigens of *M. leprae* in a highly significant proportion of patients suffering from the borderline lepromatous (BL) and LL forms of leprosy. The persistence of a small group of non-reactors in the BL-LL group may reflect the presence of diverse mechanisms of non-reactivity, including genetic factors. The demonstration of the peripheral nature of the defect in many patients, subject to modification by appropriate procedures, lays the foundation for the development of an immunotherapeutic and prophylactic vaccine for leprosy ([2, 5]).

Among the general characteristics which such an antileprosy vaccine must possess is sufficient potency to overcome the immunologic defect in leprosy patients and in that portion of the general population which is susceptible to clinical disease. We consider that the first option for such a vaccine is a mixture of BCG and *M. leprae*, which results in the liberation of immunogens as a consequence of macrophage stimulation by the BCG component, even in those normal individuals and the majority of patients who are incapable of responding to repeated injections of *M. leprae* by itself. Early foundations for this concept for a two-component vaccine were laid by Hanks and Fernández in the murine leprosy model ([9]). These investigators demonstrated that a mixture of BCG and heat-killed *M. lepraemurium* reinforced resistance to murine leprosy to a degree unobserved with the use of either mycobacterium by itself.

An important aspect of our concept of vaccination in leprosy relates to the use of available experimental models for possible vaccine evaluation. Unfortunately, the results obtained in these models cannot be extrapolated to man, since these animal models do not possess the unique immunological defect observed in man. Stanford has recently supported this viewpoint, concluding that the efficacy of a vaccine against leprosy should be tested in the only animal that is naturally susceptible to leprosy—man ([17]). It seems equally clear that final vaccine evaluation can only be carried out in areas endemic for leprosy; natural factors such as the ambient mycobacterial or mycotic flora as well as the possibility that individuals in these areas may already harbor subclinical infection with *M. leprae*, represent factors which may profoundly affect the response to a given vaccine.

Additionally, the two components of the vaccine used, heat-killed *M. leprae* and BCG, have been used extensively in intradermal injections in man for many years without producing significant secondary reactions. Therefore there was good reason to expect that a mixture of the two would be well tolerated in non-reactors to *M. leprae*.

The priority and urgency for the development of a preventive and curative vaccine for leprosy has surged to the forefront in leprosy control as a consequence of the development of drug-resistant strains of *M. leprae*. In different parts of the world, secondary resistance to sulfones in 4%–6% of patients and primary resistance in 3%–40% have been reported ([11, 16]). The development of an immunotherapeutic procedure to complement the chemotherapeutic procedures now available constitutes a step forward and might represent a decisive factor in limiting the development of drug-resistant strains of *M. leprae*.

Based on the preceding considerations, in 1973 we began the application of a mixture of *M. leprae* and BCG in a very limited group of individuals: 6 patients with LL, inactive and bacteriologically negative after prolonged treatment with sulfones; 6 patients with indeterminate leprosy; and 6 contacts, all of whom were persistently Mitsuda negative. Contacts usually received a single dose of vaccine; patients were vaccinated several times during a period of 1–2 years. As a result of careful observation during a period of almost six years, we observed significant immunological changes,

including strong and persistent positivization of the Mitsuda reaction. Since secondary phenomena were not observed, we proceeded to evaluate the activity of the *M. leprae*-BCG mixture in the following groups of persons, who represent an increasing scale of incapacity to respond to immunization:

1) Persistently Mitsuda-negative contacts, apparently healthy but weak or non-reactors to *M. leprae*.

2) Mitsuda-negative patients with indeterminate leprosy, apparently in the initial stages of disease development, but with a potential to develop lepromatous leprosy because of their condition as persistent non-reactors to the Mitsuda antigen.

3) Patients with lepromatous leprosy, bacteriologically negative after many years of treatment with sulfones. In these individuals, effective vaccination would prevent relapse and/or re-infection and the subsequent creation of new infectious foci.

4) Patients with LL or BL leprosy in different stages of activity, ranging from cases with bacteriologically positive lesions persisting after years of drug treatment to new untreated cases.

The effects of the vaccine have been evaluated by the immunological changes produced, and by clinical, histopathological, and bacteriological criteria in patients with active disease.

The majority of the patients receive chemotherapy simultaneously; a small group of patients have received immunotherapy alone because of important secondary drug reactions or evidence of bacterial resistance.

The studies reported in the first part of this paper represent an evaluation of immunotherapy in leprosy. We would re-emphasize that immunological non-reactors to *M. leprae* represent the source of new cases in endemic areas and play a fundamental role in perpetuating the endemic situation. This point will be considered in further detail in delineating a strategy for immunoprophylaxis.

CASES, METHODOLOGY AND MATERIAL

Cases

The 577 cases studied include the following groups of persons:

1) 25 contacts of lepromatous patients who were persistently Mitsuda negative, even after several vaccinations with BCG.

2) 46 Mitsuda-negative patients wtih indeterminate leprosy who presented multiple hypochromic lesions with alterations in sensitivity. These patients were Mitsuda negative after three or four applications of standard lepromin and two or three vaccinations with BCG.

3) 155 patients with borderline lepromatous or lepromatous leprosy, bacteriologically negative or with few bacilli after prolonged chemotherapy.

4) 351 patients with active BL and LL leprosy.

Methodology

All the individuals studied were given dermatologic, neurologic, ophthalmologic, and general clinical examinations. A biopsy for histopathologic evaluation was routinely stained by hematoxylin and eosin (H&E) and Fite-Faraco procedures. *In vivo* and *in vitro* immunologic tests included: skin tests with standard Mitsuda antigen and soluble protein antigen (SPA) from *M. leprae*, lymphocyte transformation to mitogens and *M. leprae*, suppressor cells, and micro-ELISA test for circulating antibodies.

The vaccine was administered by the intradermal route; 0.5 ml of the mixture is distributed in three sites in the deltoid regions and upper back. All the patients were examined at weekly intervals during the first stages of the study and subsequently as often as was necessary. During the course of the study, biopsies were taken for histopathologic study and blood samples were taken for lymphocyte transformation, ELISA and, in some cases, study of suppressor cells; these last studies were carried out in collaboration with Dr. Barry Bloom and his associates. Skin tests with SPA were performed when clinical or histopathological changes were observed, and before re-vaccination.

Material

The vaccine was composed of a mixture of *M. leprae* obtained from the tissues of experimentally infected armadillos, purified by the Draper 1/79 protocol ([7]) and heat killed by autoclaving at 121°C for 15 min, and viable BCG (Institut Pasteur, Paris, France). Each dose of vaccine contained 6×10^8 *M. leprae* in a volume of 0.4 ml

TABLE 1. *Immunotherapy with a mixture of* M. leprae *plus BCG in leprosy groups studied and changes obtained.*

Classification and period of vaccination (mo)	No.	Skin test reactivity		Clinical and histopathological changes		
		SA[a] positive (%)	Mitsuda positive (%)	Reduced infiltration (%)	Reversal reaction (%)	Total (%)
Active BL/LL						
6	18	0	—	0	8	6
18	74	20	—	27	20	47
>19	259	38	—	43	27	71
Inactive BL/LL						
6	3	67	—			
18	39	46	—			
>19	113	63	—			
Indeterminate						
6	2	100	—			
18	12	83	75			
>19	32	97	88			
Contacts						
6	25	100	84			

[a] SA = soluble antigen.

and 0.1 ml of BCG of variable concentration, depending upon the prior cutaneous response to purified protein derivative (PPD).

RESULTS

Table 1 shows the distribution of patients, period of observation, changes in the 48-hr skin test reactivity to SPA and clinical changes. Of the 155 patients with inactive BL and LL, 59% became SPA-positive; 32% of the 351 patients with active BL and LL became positive. Positivity was more frequent in those patients who had been vaccinated more than six times.

The group of 25 persistently Mitsuda-negative contacts represents one of the most interesting groups with regard to the potential use of this vaccine in the immunoprophylaxis of leprosy. All showed a positivization of their reactions to SPA after one or, rarely, two vaccinations. All who have been tested were strongly Mitsuda positive after vaccination. Some of these individuals might be suspected of harboring the infection which would become manifest as tuberculoid or borderline tuberculoid leprosy in the presence of cell-mediated immunity, but no such case has appeared.

In the group of 46 Mitsuda-negative indeterminate patients studied, important changes have occurred in clinical, histopathologic, and immunologic characteristics. The clinical and histopathologic changes were observed after two, three, or more vaccinations given at intervals of two to three months. Repigmentation of hypochromic lesions was observed after two or three years. An eruption formed by multiple small papules occurred often; in a single case, a typical tuberculoid plaque appeared which regressed after six months. Histopathologic study of the papular lesions showed a follicular tuberculoid structure; the papules disappeared simultaneously after three or four months. There were 43 positive reactions to SPA and 37 to Mitsuda antigen. Four or more vaccinations were required to produce the changes described in those patients who showed progressive evolution toward lepromatous leprosy prior to vaccination, as indicated by the presence of bacilli at sites far from the lesions (earlobes, knees).

As expected, it has been more difficult to induce immunologic changes in lepromatous or borderline lepromatous patients, even in those who were free of clinical lesions after prolonged chemotherapy. In this latter group of 155 individuals, 91 have become immunologically reactive to SPA after three or more vaccinations (59%).

The changes observed in the group of ac-

tive BL and LL patients who have received three or more vaccinations are of extraordinary interest. The clinical pathological changes include reversal reactions characterized by formation of nodules and plaques superimposed on chronic lesions, reactivation of lesions with sharper definition of their borders and, in other cases, progressive regression of the lesions.

Parallel to the clinical changes mentioned, important histopathological changes have also occurred. These changes are of two types, which depend upon the degree of vacuolization of the macrophages and the characteristics of the bacterial population of the granuloma. In lesions formed by highly vacuolated, bacilliferous macrophages, the most striking observation after vaccination was the accumulation of numerous, relatively small macrophages and lymphoid infiltration into the granuloma. These new cells may exert a cytotoxic "killer" function against the original cells, or act as scavenger cells. In other cases in which the macrophage compromise appeared to be less severe, various degrees of epithelioid differentiation and more abundant lymphoid cell infiltration were observed. Macrophage activation by lymphokines may be a more important mechanism of bacterial elimination in these lesions.

Out of a total of 351 active BL and LL patients vaccinated three or more times, 62% have shown some or all of the clinical and histopathological changes described earlier. One hundred-thirteen have become SPA positive. The majority required five or more vaccinations to show these changes. The clinical, histopathological, and immunological changes that were observed and that were designated as reversal reactions were of an intensity and consistency sufficiently strong to consider that they have shifted in the spectrum from LL to BL-BB and BT.

The histopathological changes which occurred in these patients have recently been evaluated in an international workshop by a group of six histopathologists: Drs. Raul Negrao Fleury and Rene Garrido Neves, Brazil; Drs. Chapman H. Binford and Wayne M. Meyers, U.S.A.; Dr. A. Colin McDougall, England, and Dr. Oscar Reyes, Venezuela. The preliminary evaluation of these results are shown in Table 2 and Figure 1. A biopsy taken prior to immunotherapy and three or four biopsies taken subsequently were analyzed in terms of an arbitrary point scale (LL = 90–100, BL = 50–69, BT = 30–49) and results are expressed as the average value of five to six independent observations. These results revealed modifications in classification (reversal reactions) in 90.5% of the patients initially classified as LL and 83.3% of the BL patients. These results will be fully described and analyzed by the group of histopathologists in the near future.

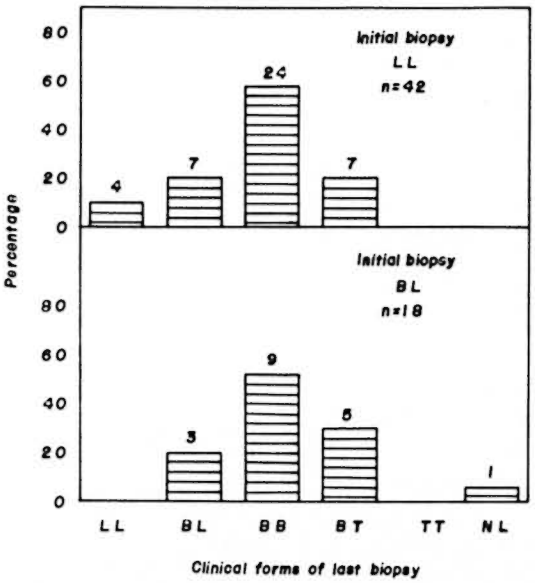

FIG. 1. Histopathological demonstration of reversal reactions induced by immunotherapy.

Secondary reactions

Massive transformation of undifferentiated macrophagic granulomata into granulomata with epithelioid differentiation and infiltration by lymphoid elements was accompanied by an important reduction in the bacterial population; in these cases we have observed fever and general malaise, easily controlled by small doses of corticosteroids.

An observation of great importance was the almost complete absence of reactions in nerve trunks. If it were not for this phenomenon, vaccine therapy in bacilliferous leprosy would not be possible. We have observed severe neuritis in only 4 of the total of 351 cases of active BL and LL studied, and moderate reactions in 19 others. These reactions consisted of pain in cubital and

TABLE 2. *Modifications of diagnostic classification in patients treated with immunotherapy, according to initial histopathological classification of LL or BL.*

Group	Initial biopsy	Biopsies after immunotherapy			Biopsy with greatest modifications
	1	2	3	4	
LL patients					
Number studied	42	42	38	22	42
Average score	95.6	68.9	63.6	71.3	61.4
Classification[a]	LL	BB	BB	BL	BB
BL patients					
Number studied	18	18	18	10	18
Average score	82.2	64.0	60.3	51.0	54.8
Classification	BL	BB	BB	BB	BB

[a] See text for the relationship between score and classification.

sciatic nerves that were enlarged at the time immunotherapy was begun. They were rapidly controlled by corticosteroids and left no permanent sequellae.

Recently we have observed transitory jaundice in two cases of BL with reversal reaction. These reactions were easily controlled with 8 mg/day of triamcinolone administered for a week. Edema of the dorsum of the hands, feet, and lower legs have been observed in five cases, and was controlled with doses of 4 mg–8 mg corticosteroid daily for periods of four to six weeks.

In summary, the secondary reactions observed during the course of these studies have been moderate and easily controlled by appropriate treatment. They do not compare unfavorably with the reactions observed during the course of conventional chemotherapy.

Immunoprophylaxis

Having presented our results in the use of a vaccine against leprosy in patients and contacts, we would like to refer briefly to our concept of the basis for immunoprophylaxis in leprosy.

The strategy for leprosy control based on treatment of the sources of infection is basically sound, but the possibilities of practical success are very limited. The chronic nature of the disease requires such prolonged treatment that these programs are frequently abandoned by the patients. In the majority of countries where leprosy is endemic, the prevalence of the disease has not been significantly reduced. As Dr. S. G. Browne has recently pointed out, in some aspects, the situation today is comparable to that which existed at the beginning of the 1940s, before the era of the sulfones. A new strategy, based on the development of a highly efficient, low-cost, risk-free preventive vaccine, would constitute a decisive element in control or erradication programs in those areas of the world where leprosy constitutes an important public health problem.

The bases for immunoprophylaxis as we understand it at present can be summarized as follows:

1) The population susceptible to leprosy, especially in its progressive forms, represents a very small proportion of the general population. This fact, plus the possible high cost of producing a vaccine based on *M. leprae* purified from experimentally infected armadillos, justify a selective approach to vaccination, limited to high risk populations, that is, contacts.

2) The high-risk population can be identified by two criteria—epidemiological and immunological. Through the first, we identify contacts around active leprosy cases. In Venezuela, we estimate an average of five intradomicilliary and 45 extradomicilliary contacts per case, on the basis of previous epidemiological surveys. The interest in extradomicilliary contacts is based on the fact that 75% of new leprosy cases have been found among this group.

The immunological criterion used to identify high-risk contacts is a negative reaction towards soluble *M. leprae* antigen. Supposedly all contacts have been exposed to infection by *M. leprae* and those with

normal immunological reactivity should have developed delayed hypersensitivity towards this organism.

3) More than 25 years ago, Dharmendra and Chatterjee studied the incidence of leprosy in individuals living in endemic areas of India. This study revealed that 55% of the lepromatous cases detected 15 to 20 years after the initial survey with Mitsuda antigen occurred in that 2.3% of the population which remained persistently negative after repeated lepromin injections; the other 45% of the lepromatous cases occurred in initially Mitsuda-negative contacts who were not repeatedly stimulated with lepromin. These and later studies, as well as the discouraging results of large scale trials with BCG, indicate that the use of either *M. leprae* or BCG alone does not offer much hope of an adequate vaccine.

The favorable results obtained in the immunotherapy of leprosy with a mixture of *M. leprae* and BCG, plus the persistence of the immunological conversion seen in Mitsuda-negative contacts during an observation period of several years, indicate that this combination can represent a highly efficient vaccine.

4) The only absolute criterion to determine the efficacy of a preventive vaccine would be the incidence of new cases of leprosy, and the evaluation would depend on a five- or ten-year observation period. In any case, observations during the immunotherapy trial indicated that the induction of an immunological conversion of skin reactivity towards soluble *M. leprae* antigen can be useful in evaluating the response to the vaccine in terms of percentage of positive reactors and persistence of conversion.

Preliminary data obtained in an immunoprophylaxis trial carried out in two western states of Venezuela which are highly endemic for leprosy are extremely interesting. In the first stage of this trial we identified a total of 2659 contacts in two work areas in Apure and Tachira states; 293 of these contacts were intradomicilliary. We examined them all, clinically and neurologically, and applied skin tests with 2 units of PPD and 0.5 μg or 1.0 μg of soluble *M. leprae* antigen. We also studied circulating antibodies through a micro-ELISA test. Skin reactivity towards these soluble antigens is shown in Figure 1. We found 21.8% of "non-reactors" in the population 12 years old or older when using a criterion of induration of 9 mm or less at 48 hr. The non-reactors were divided in two groups: one to be vaccinated with the same mixture of autoclaved *M. leprae* and BCG used for immunotherapy and the other, the control group, only with BCG.

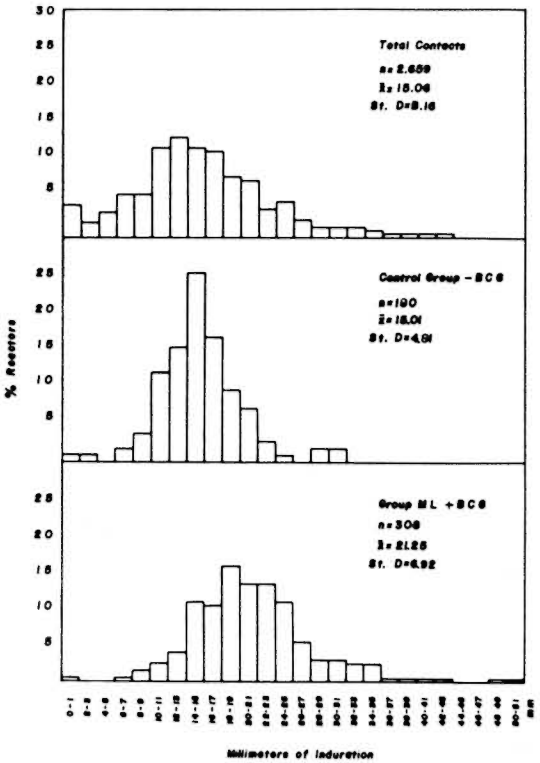

FIG. 2. Reactivity to soluble antigen in leprosy contacts and induced by vaccination with BCG or with the mixture of *M. leprae*-BCG in contacts initially negative 60 days after vaccination control in the Venezuelan states of Apure and Tachira, 1981.

Two months after vaccination both groups were again tested with *M. leprae* soluble-antigen. As we can see in Figure 2, a high percentage of both groups became positive. In the group of 305 persons vaccinated with the mixture *M. leprae*-BCG, we saw an average induration of 21.22 mm at 48 hr with soluble antigen; 56% of this group gave strong reactions (20 mm of induration or more) and only 1.9% persisted as "non-reactors" with reactions of 9 mm or less of induration. On the other hand, the average induration in the group of 180 persons vac-

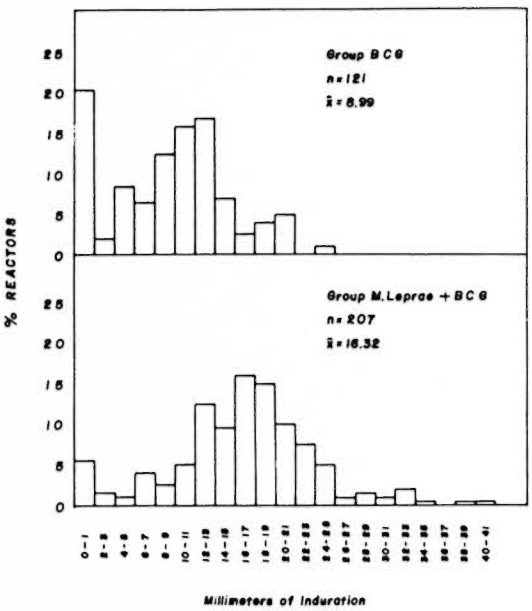

FIG. 3. Reactivity to soluble antigen in leprosy contacts and induced by vaccination with BCG or with the mixture of *M. leprae*-BCG in contacts initially negative eight months after vaccination control in the Venezuelan states of Apure and Tachira, 1982.

cinated with BCG alone was 15.02 mm; only 14% gave reactions of 20 mm or more and almost 8% persisted as "non-reactors." This initial evaluation indicated that the *M. leprae*-BCG mixture induced an immunological conversion towards soluble antigen clearly superior to that induced by BCG alone.

The difference in reactivity towards soluble antigen in both groups became much more evident at eight months after vaccination (Fig. 3). In the control group vaccinated with BCG, more than 60% presented reactions of 9 mm or less of induration (average of the whole group, 7.84 mm). This fact shows that BCG induced not only weak, but also short-lived reactivity. The average of induration in the group vaccinated with the mixture was 15.14 mm at eight months, and 15% had reactions of 9 mm or less. These results at eight months supported the initial conclusion obtained with the results at two months. The future stages of this trial contemplate the repetition of the clinicodermatological examination and skin tests with soluble *M. leprae* antigen at yearly intervals over five years and less frequent examinations in the next five years. The preliminary results have already become the basis for the protocol of a much larger trial in Venezuela, which will include the study of 61,000 contacts.

The results of the immunotherapy and immunoprophylaxis programs developed in Venezuela offer the hope of new methods for antileprosy campaigns at a time when the alarming increase of sulfone resistance makes it necessary to intensify the search for a new approach to solve this problem.

Some comments are indicated on the group of persons who after being vaccinated with the mixture appear as negatives (0 mm–9 mm) or weakly positive (10 mm–14 mm) after an intradermal test with soluble antigen. A not yet determined percentage of these negative persons have high titers of circulating antibodies with the micro-ELISA test. This situation is similar to that seen in patients with low resistant forms of leprosy (LL, BL, and some Mitsuda-negative, indeterminate patients), and we have considered the possibility that this group may require more than one vaccination in order to obtain favorable immunological changes. According to these last considerations, the above mentioned group of non-reactors would be the root of the leprosy endemia. Therefore, the schedule of antileprosy campaigns could be the following: contact population divided into reactors and non-reactors to soluble antigen, vaccination with the *M. leprae*-BCG mixture and later revaccination of the persistently non-reacting group. With this approach the antileprosy campaign would be secondary to the immunotherapy of "non-reactors" after the first dose of vaccine since, by producing immunological changes in this population group, we would prevent the creation of new infected foci and, therefore, the maintenance of the endemia.

REFERENCES

1. BULLOCK, W. E., JR. and FASAL, P. J. Studies of immune mechanisms in leprosy. III. The role of cellular and humoral factors in impairment of the *in vitro* immune response. J. Immunol. **106** (1971) 888–899.
2. CONVIT, J., ARANZAZU, N., PINARDI, M. E. and ULRICH, M.. Immunological changes observed in indeterminate and lepromatous leprosy patients and Mitsuda-negative contacts after the inocula-

tion of a mixture of *Mycobacterium leprae* and BCG. Clin. Exp. Immunol. **36** (1979) 214–220.

3. Convit, J., Avila, J. L., Goihman-Yahr, M. and Pinardi, M. E. A test for the determination of competency in clearing bacilli in leprosy patients. Bull. WHO **46** (1972) 821–826.
4. Convit, J., Pinardi, M. E., Rodriguez-Ochoa, G., Ulrich, M., Avila, J. L. and Goihman-Yahr, M. Elimination of *Mycobacterium leprae* subsequent to local *in vivo* activation of macrophages in lepromatous leprosy by other mycobacteria. Clin. Exp. Immunol. **17** (1974) 261–265.
5. Convit, J., Ulrich, M. and Aranzazu, N. Vaccination in leprosy—observations and interpretations. Int. J. Lepr. **48** (1980) 62–65.
6. Deo, M. G., Bapat, C. V., Bhalerao, V., Chaturvedi, R. M., Bhatki, W. S. and Chulawala, R. G. Antileprosy potentials of ICRC vaccine. A study in patients and healthy volunteers. Int. J. Lepr. **51** (1983) 540–549.
7. Draper, P. In: Problems related to purification of *M. leprae* from armadillo tissues and standardization of *M. leprae* preparations. Report IMMLEP, Annex 1, Feb. 1979.
8. Godal, T., Rees, R. J. W. and Lamvik, J. O. Lymphocyte-mediated modification of blood-derived macrophage function *in vitro*; inhibition of growth of intracellular mycobacteria with lymphokines. Clin. Exp. Immunol. **8** (1971) 625–637.
9. Hanks, J. H. and Fernandez, J. M. M. Enhancement of resistance to murine leprosy by BCG plus specific antigen. Int. J. Lepr. **24** (1956) 65–73.
10. Hirchberg, H. The role of macrophages in the lymphoproliferative response to *Mycobacterium leprae in vitro*. Clin. Exp. Immunol. **34** (1978) 46–51.
11. Increase in prevalence of leprosy caused by dapsone-resistant *Mycobacterium leprae*. Morbid. Mortal. Weekly Rep. **30** (1982) 637–638.
12. Leiker, D. L. Studies on the lepromin test. I. The influence of the bacillary and tissue components in dilutions of lepromin. Int. J. Lepr. **29** (1961) 157–167.
13. Mehra, V., Mason, L. H., Fields, J. P. and Bloom, B. R. Lepromin-induced suppressor cells in patients with leprosy. J. Immunol. **123** (1979) 1813–1817.
14. Mehra, V. L., Talwar, G. P., Balakrishnan, K. and Bhutani, L. K. Influence of chemotherapy and serum factors on the mitogenic response of peripheral leucocytes of leprosy patients to phytohaemagglutinin. Clin. Exp. Immunol. **12** (1972) 205–213.
15. Myrvang, B., Godal, T., Ridley, D. S., Froland, S. S. and Song, Y. K. Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy. Clin. Exp. Immunol. **14** (1973) 541–553.
16. Nath, I., van Rood, J. J., Mehra, N. K. and Vaidya, M. C. Natural suppressor cells in human leprosy: The role of HLA-D-identical peripheral lymphocytes and macrophages in the *in vitro* modulation of lymphoproliferative responses. Clin. Exp. Immunol. **42** (1980) 203–210.
17. Pearson, J. M., Rees, R. J. W. and Waters, M. F. R. Sulphone resistance in leprosy. A review of one hundred proven clinical cases. Lancet **2** (1975) 69–72.
18. Stanford, J. L. A vaccine for leprosy. Lepr. Rev. **47** (1976) 87–91.
19. Talwar, G. P. and Fotedar, A. Two candidate antileprosy vaccines—current status of their development. Int. J. Lepr. **51** (1983) 550–552.

INTERNATIONAL JOURNAL OF LEPROSY

Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Antileprosy Potentials of ICRC Vaccine. A Study in Patients and Healthy Volunteers[1]

Madhav G. Deo, Chandrashekar V. Bapat, Vijaya Bhalerao, R. M. Chaturvedi, Waman S. Bhatki, and Ramesh G. Chulawala[2]

Immunoprophylaxis, which is aimed at enhancing host immune defense mechanisms, is an established modality for control of microbial diseases. Usually attenuated organisms, that have lost pathogenicity but retained antigens that induce protective immunity, are used in vaccine preparation. Alternatively, nonpathogenic, live or killed, antigenically crossreactive microbes could also be used. The latter approach is quite attractive, particularly for immunoprophylaxis of leprosy, because *Mycobacterium leprae*, the causative agent, has yet to be cultivated. Large quantities of *M. leprae* could be, however, obtained from armadillos, in which the bacilli grow profusely ([22]). In fact a vaccine containing killed *M. leprae* A (armadillo grown) and BCG has been successfully developed by Convit, *et al.* in Venezuela ([10]).

Among the cultivable organisms, trials with BCG have given conflicting results with reference to its protective efficacy in leprosy ([35]). Three years ago we developed a vaccine from ICRC bacillus killed by gamma irradiation ([11]). The bacillus, which is a slow-growing *Mycobacterium*, has been repeatedly cultivated from human lepromata since 1958 ([1]). It lacks antigen 2, which is among the most stable and reproducible antigen of *M. leprae*, and it lacks some determinants of antigen 4 that are specific for *M. leprae* (Closs, O., personal communication). On the basis of sero-antigens, the ICRC bacillus has been shown to belong to the *M. avium-intracellulare* group of organisms ([11]). However, sero-antigens have little role to play in protective immunity against leprosy. Studies carried out, both in man (patients as well as healthy subjects) and laboratory animals, have shown that ICRC crossreacts with *M. leprae* antigens that are involved in cell-mediated immunity (CMI) ([16, 17]) which is the host's major defense against the organisms ([18]). This stimulated us to use ICRC in vaccine preparation.

The lepromin (Mitsuda) reaction is consistently negative in lepromatous leprosy (LL) patients ([30]). The anergy is highly specific to *M. leprae* antigens; responses to other antigens are by and large unaffected ([15]). Even after prolonged drug therapy, the immune defect is not corrected and the patients continue to harbor bacilli in the tissues ([5, 18, 32]). These patients may require additional immune stimulus, possibly by way of a vaccine, in order to overcome the immune defect. In a normal population, even in endemic areas, a small group of individuals exhibit persistent lepromin negativity ([28]). They run a high risk of contracting the multibacillary forms of leprosy ([12]). A vaccine which is able to bring about lepromin conversion in LL patients is also expected to do so in these individuals. With these objectives, we have tried the ICRC vaccine both in leprosy patients and lepromin negative residents in an endemic area.

PATIENTS, HEALTHY VOLUNTEERS, MATERIALS AND METHODS

The hospital-based studies on the patients were carried out in Acworth Leprosy

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] M. G. Deo, M.B., B.S., M.D., Ph.D., Research Director, and C. V. Bapat, Ph.D., Head, Cell Biology Division, Cancer Research Institute, Parel, Bombay, India. V. Bhalerao and R. M. Chaturvedi, Department of Preventive and Social Medicine, G. S. Medical College, Parel, Bombay, India. W. S. Bhatki, M.B., B.S., D.V.D., D.P.E., Medical Officer, and R. G. Chulawala, B.Sc., Research Assistant, Acworth Leprosy Hospital, Wadala, Bombay, India.

TABLE 1. *Plan of the study.*

Patients/volunteers	No.
LL (vaccinated)	71
LL (controls)	19
BB/BL (vaccinated)	11
BB/BL (controls)	10
Lepromin-negative household contacts of LL (vaccinated)	12
Lepromin-negative noncontacts (vaccinated)	20
Lepromin-negative nonvaccinated residents (controls)	17
Total	160

Hospital, Bombay, India. The field studies, on healthy volunteers, were conducted at a primary health care center established by the G. S. Medical College in Malwani (population 45,000), a suburb of Bombay. The plan of the study is depicted in Table 1.

Hospital-based investigations

A total of 90 LL and 21 BB/BL patients, 10–65 years of age, participated in the study. The patients were diagnosed on the basis of clinical presentation, Bacteriologic Index (BI) and, in many cases, skin biopsies. They were classified according to the Ridley and Jopling scale ([33]). All were on dapsone (DDS) for a period of 3–25 years. In addition, some also received rifamycin. Depending on the duration of the treatment, the BI in the LL patients varied from 5+ to 1+.

Seventy-one LL and 11 BB/BL patients received 0.1 ml of the vaccine containing 27 μg–67 μg protein (50 μg equivalent to 1×10^9 bacilli) intradermally in the left deltoid region. The rest (19 LL and 10 BB/BL who received saline, that was used as the vehicle) served as controls. The vaccine was prepared from ICRC strain C-44 that was isolated from a lepromatous patient in 1969. Details of the vaccine preparation, standardization of doses, etc., have been described elsewhere ([11]).

The Mitsuda reaction was carried out using lepromin A (Lot No. AB-22) generously provided by Dr. R. J. W. Rees, Head, Laboratory for Leprosy and Mycobacterial Research, Medical Research Council, London, England. Each patient received intradermally 0.1 ml of lepromin containing 1.6×10^8 bacilli/ml in the left forearm. Skin reaction (induration) was measured 3–4 weeks later. Induration of more than 3.0 mm denoted a positive reaction. As expected, all patients were lepromin negative before vaccination. Lepromin reaction was repeated between 4–10 months after vaccination in all patients. In 26 vaccinated LL patients, the test was carried out the third time between 18–30 months post-vaccination.

To get an idea of the comparative responses, in some vaccinated LL patients (both lepromin converted and nonconverted) a study of the skin reaction to lepromin and ICRCin, a Mitsuda-type of particulate antigen of ICRC, was simultaneously carried out. Both antigens were given at the dose of 1.6×10^7 bacilli/patient.

Field studies

Seventy-three household contacts, aged 5–50, from 23 families residing in Malwani, volunteered for the study. Each family had at least one index case of the multibacillary forms (BL/LL) of leprosy. Careful and thorough clinical examination revealed no evidence of disease in the contacts. Mitsuda reaction was conducted on all patients and their family contacts using 0.1 ml of the antigen containing 4×10^7 bacilli/ml. It is known that the lepromin test by itself could act as a micro-vaccination and could result in conversion in some negative individuals. In order to reduce such a possibility, a dose lower than that used in patients was purposely employed in the field studies. Lepromin A was obtained through the kind courtesy of Dr. W. F. Kirchheimer, National Hansen's Disease Center, Carville, Louisiana, U.S.A. with the assistance of the World Health Organization (WHO).

Among the contacts, 28 were Mitsuda negative and 12 of them, aged 10–45 years, volunteered for vaccination. They received 0.1 ml of vaccine containing 0.25 μg protein (equivalent to 5×10^8 bacilli) intradermally in the left deltoid region.

The pattern of lepromin reactivity was also studied in the 400 volunteers between 10–18 years of age in the general population. In 17 of them (8 males and 9 females) who were lepromin negative, the test was repeated to assess the effects, if any, of the first test on their subsequent lepromin reactivity.

To get an idea of a minimum effective

TABLE 2. *Vaccine-induced ENL and lepromin conversions in different forms of leprosy.*

Group	Type of leprosy	Vaccine dose (μg protein)	No.	Age range (yr)	Sex		Early (10–30 days)		Late (4–10 months)	
					M	F	Ulcer	ENL	Mitsuda positive	
									Total	%
I	LL	27–67	71	10–65	65	6	71	21	41	57.7
II	LL (Control)	Saline only	19	10–65	17	2	Nil	Nil	Nil	Nil
III	BB/BL	27–67	11	31–60	9	2	11	Nil	10	91
IV	BB/BL (Control)	Saline	10	22–56	10	0	Nil	Nil	1	10

dose, 20 lepromin-negative residents, aged 12–18 years, received the vaccine in the dose of 0.8 μg/person (equivalent to 1.6×10^7 bacilli), which is the highest concentration at which *M. leprae* is used as the Mitsuda antigen. In order to bring more objectivity, lepromin reactivity in different groups was also assessed by experienced "blind" observers.

Histopathology

Biopsies were obtained from the lesions in the upper extremities and the back of LL patients before and after vaccination, as well as sites of lepromin tests in a number of converted patients. Biopsies were fixed in Zenker-formol, cut at 5 μ, and stained routinely with hematoxylin and eosin (H&E) and Fite's stain for acid-fast bacilli (AFB).

RESULTS

Patients

Vaccination hardly produced any acute local or systemic reaction. However, 2–3 weeks after vaccination an ulcer developed at the local site associated with enlargement of regional lymph nodes in all patients (Table 2). The ulcer healed with local treatment. The vaccine was well tolerated and did not produce any untoward systemic effects. Erythema nodosum leprosum (ENL), which occurred in 3–4 weeks after vaccination, was observed in 30/71 (42.2%) LL patients, who were mostly high index cases (BI 3+ or more). Lepromin conversion was observed in 57.7% and 91% of LL and BB/BL patients, respectively, between 4–10 months of vaccination. During the same period only 10% control BB/BL but none of the unvaccinated LL exhibited lepromin conversion. None of the converted patients developed fresh nerve lesions. No differences were observed between males and females, and for subsequent analysis they were treated as one group.

Analysis of lepromin conversion in the LL patients in relation to different variables, such as the age of the patient, duration of treatment, batch of vaccine, etc., is shown in Table 3. Conversion appeared to be independent of any of these factors.

TABLE 3. *Rate of conversion in LL patients in relation to different parameters.*

Parameters	No. patients	Lepromin conversion	
		Total	%
Age (yr)			
Below 20	15	8	53
21–50	45	28	62
51–65	11	5	45
Treatment			
DDS only	61	37	61
DDS + rifamycin	10	4	40
Bacillary Index			
4 to 5+	45	30	66
3+ and below	26	11	42
Dose of vaccine (μg)			
27–49	27	11	41
50–67	44	30	68
Batch of vaccine with dose (μg)			
V_1 (45)	4	1	25
V_2 (67)	11	7	64
V_3 (34–50)	12	7	59
V_4 (27–45)	13	8	61
V_5 (30–60)	12	9	75
V_6 (30–60)	12	6	50
V_7 (30–60)	75	1	20
V_{15} (30–60)	2	2	100

FIG. 1. Pre-vaccination biopsy of a skin lesion of a lepromatous patient on long-term dapsone (DDS) therapy. It shows more or less normal epidermis, a clear subepidermal zone, and a focal histocytic infiltration in the dermis. Very few lymphocytes are seen (H&E ×90).

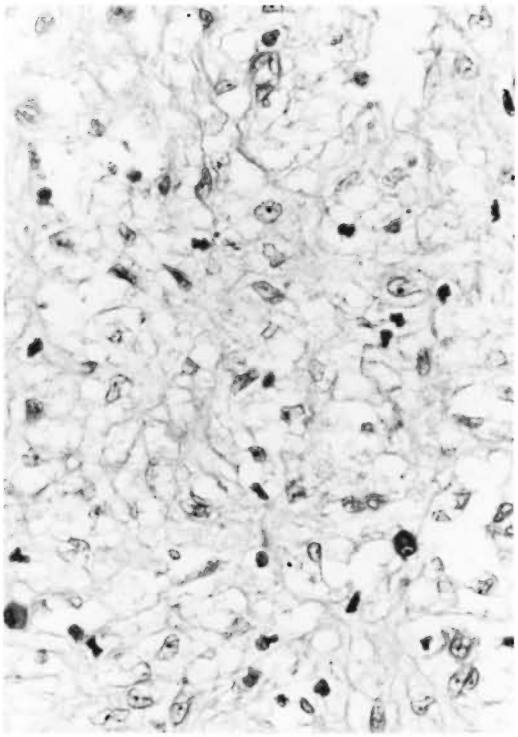

FIG. 2. High power magnification of Figure 1 to show vacuolated foamy macrophages. The lymphocytes are conspicuous by their absence (H&E ×440).

In a number of lepromin converted LL patients significant changes were observed in the morphology of the lesions. There was loosening of the granuloma and a reduction in the number of macrophages which were vacuolated and foamy. The granuloma also showed infiltration with lymphocytes and a reduction in the tissue bacillary load. "Reversal" reaction with upgrading of the lesion has been observed, so far, in five patients (Figs. 1–4).

Table 4 depicts the results of the third lepromin test carried out on 26 LL patients who were vaccinated in 1979–1980. Persistent conversion was observed in 85.7% (12/14). Further, five out of 12 (41.6%), who were initially nonresponsive, now developed a positive reaction. Thus, an increasing number of LL patients exhibited conversion over time.

It is evident from Table 5 that irrespective of the status of lepromin reaction, patients exhibited strong positive responses to ICRCin.

Healthy volunteers

The pattern of lepromin reactivity in contacts and noncontacts (general population) is shown in Table 6. Lepromin positivity was very low below ten years of age. The pattern of reactivity between 10–20 years

TABLE 4. *Persistence of post-vaccination Mitsuda response in LL patients.*

No. patients[a]	Post-vaccination Mitsuda reaction at 4–10 months (no.)		Subsequent status at 18–30 months			
			Positive		Negative	
			No.	%	No.	%
26	Converted (positive)	14	12	85.7	2	14.3
	Nonconverted (negative)	12	5	41.7	7	53.3

[a] Vaccinated in 1979–80. Lepromin positive at 4–10 months = 14/26 (53.8%). Lepromin positive at 18–30 months = 17/26 (65.4%).

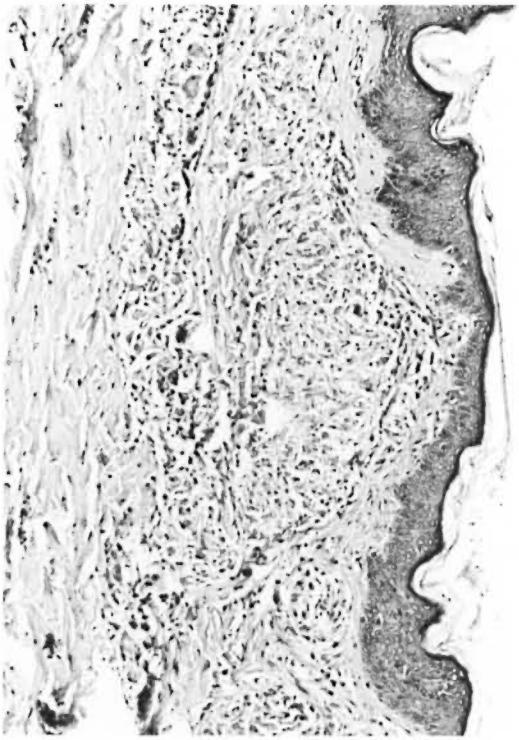

FIG. 3. Biopsy of a "reversal" site in the same patient ten months after vaccination. The epidermis is normal. The dermis exhibits granuloma containing predominantly epithelioid cells, a few giant cells, and a moderate number of lymphocytes (H&E ×110).

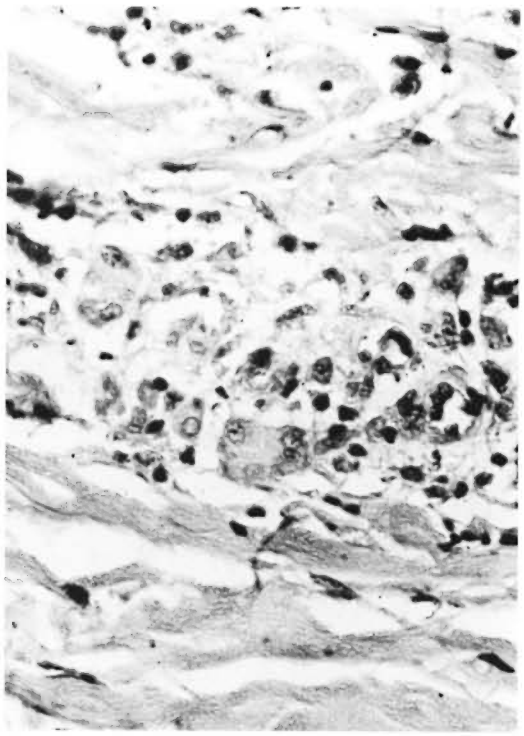

FIG. 4. High power magnification of Figure 3 to show cellular details of the granuloma (H&E ×440).

was similar in contacts and noncontacts (Table 6).

All vaccinated individuals developed a local response, including the lymph node enlargement, similar to that observed in the patients. No systemic untoward reaction was observed. Vaccination resulted in conversion of 92% (11/12) of the volunteers (Table 7). Lepromin-negative, nonvaccinated individuals, on the other hand, exhibited no conversion as a result of the first lepromin test (Table 8). An interesting phenomenon was observed in two contacts and two patients. Their pre-vaccination lepromin reaction was 0 mm–1 mm. Three weeks post-vaccination, the patients started developing some induration at the site of the first test. By the sixth week the induration had reached 3 mm–6 mm in size. Their post-vaccination lepromin reactions were not only strongly positive but in some there was even ulceration (Table 9).

The results of the low-dose vaccination in 20 lepromin-negative residents are shown in Table 10. The conversion rates were not only low (45%) but the magnitude of the

TABLE 5. *Post-vaccination Mitsuda reaction to lepromin and ICRC in "nonresponders" and "responders" (lepromin converted).*[a]

Nonresponders			Responders		
Case no.	Lepromin (post-vaccination)	ICRCin	Case no.	Lepromin (post-vaccination)	ICRCin
49	0	13.0	44	5.0	8.0
50	0	9.5	46	4.0	10.0
51	0	10.0	40	3.5	9.0
59	0	9.5	52	4.0	14.0
60	0	10.5	54	5.0	9.0
61	0	10.4	56	7.0	14.0

[a] Skin reaction (induration) in mm diameter. All patients were males and were lepromin (Mitsuda) negative prior to vaccination.

TABLE 6. *Lepromin activity in household contacts and noncontacts.*

Groups	Sex	Total	Lepromin (Mitsuda) Reaction							
			Less than 3 mm (negative)		3.5–5 mm		5.5–7 mm		More than 7 mm or ulcer	
			No.	%	No.	%	No.	%	No.	%
Household contacts										
Children 5–9 yr	M	9	7	77.8	2	22.2	—	—	—	—
	F	8	6	75.0	2	25.0	—	—	—	—
Adolescents and young adults 10–20 yr	M	16	3	18.7	11	68.8	2	12.5	—	—
	F	17	6	35.3	9	52.9	2	11.8	—	—
Adults >20 yr	M	7	1	14.3	5	71.4	—	—	1	14.3
	F	16	5	31.2	9	56.2	2	12.6	—	—
Noncontacts										
Adolescents and young adults 10–20 yr	M	223	53	23.8	110	49.3	52	23.3	8	3.6
	F	177	54	30.5	83	46.9	35	19.8	5	2.8

TABLE 7. *Lepromin conversion in negative contacts.*

No.	Name	Age	Sex	Mitsuda Reaction (mm)	
				Pre-vaccination	Post-vaccination
1	N	10	F	0	0
2	D	15	F	1	6[a]
3	H	10	M	0	5
4	R	28	F	1	7[a]
5	HK	46	M	1	7[a]
6	SK	46	F	0	5[a]
7	S	11	M	0	6[a]
8	RB	15	M	0	7[a]
9	N	25	F	1	5
10	DP	10	F	0	5
11	AB	45	F	0	6
12	NB	10	F	0	5
Total conversions = 11/12 (91%):			Males = 4/4 (100%)		Females = 7/8 (87.5%)

[a] Ulcer.

response was also smaller as compared to the response seen in household contacts given the higher dose (Table 7).

TABLE 8. *Effects of lepromin test on Mitsuda reaction in noncontacts.*

Volunteers			Mean Mitsuda reaction (range)	
Sex	Age	No.	1st test (mm)	2nd test (mm)
M	10–18	8	1.7 (0–2.5)	1.30 (0–2.5)
F	10–18	9	1.4 (0–3)	1.0 (0–3)

Measurements made by a "blind" observer (Dr. M. D. Gupte, Professor, Preventive and Social Medicine Department, Wardha, India) are shown in Table 11. The two readings, which were taken at 48-hour intervals, show excellent concordance.

DISCUSSION

Immunoprophylactic efficacy of a vaccine has to be ultimately assessed in field trials by its capacity to prevent the disease in susceptible subjects. Leprosy has a very long incubation period, and therefore the trials will have to be carried out for several years in order to obtain any meaningful data. Before initiating field trials it would be essen-

TABLE 9. *Post-vaccination reactions at the two sites of lepromin tests.*

Name (category)	Age	Sex	Induration at site of lepromin tests (mm)		
			First test (Left forearm)		Second test[a] (Right forearm)
			Pre-vaccination	Post-vaccination (6 weeks)	Post-vaccination (8 weeks)
H (contact)	10	M	0	3	5
SK (contact)	46	F	0	4	5[b]
IU (patient)	50	M	0	6	9
IB (patient)	30	F	1	6	9[b]

[a] Second test was performed eight weeks after vaccination and read four weeks later.
[b] Ulcer.

tial to establish that the vaccine is able to enhance protective immunity, and the determinants of protective immunity are often difficult to define. Pioneering work of Mackaness has established the importance of CMI in the handling of intracellular parasites ([23]). CMI is also the main defense mechanism against *M. leprae* and the circulating antibodies have little role ([18, 27]). Among the laboratory parameters of CMI, it appears that the lymphocyte transformation test correlates well with hypersensitivity ([4]). Relationship of the SPA test, a 48-hour skin reaction to soluble protein antigens (SPA) of *M. leprae* developed by Convit, *et al.* ([9]), to protective immunity is yet to be established. Mitsuda-type skin reaction to particulate antigens of the leprosy germs, on the other hand, has good correlation with the capacity of the host to handle *M. leprae*. Thus, within the leprosy spectrum the paucibacillary forms are associated with a positive reaction; while the patients suffering from multibacillary forms are consistently lepromin negative ([18]). As mentioned earlier, epidemiological studies likewise indicate that Mitsuda-negative individuals, in endemic areas, run a very high risk of contracting the multibacillary forms of leprosy ([12]).

TABLE 10. *Pre-vaccination and post-vaccination lepromin reaction in noncontacts.*

Name	Age	Sex	Lepromin reaction (mm)	
			Pre-vaccination	Post-vaccination[a]
NV	13	M	0	4
PV	14	M	1	7
KS	13	F	1	4
AS	14	F	1	5
Sumant	15	F	1	7
Sawant	16	F	1	5
DK	17	F	1	6
SA	13	F	1	4
S	14	F	0	5
SK	14	F	1	3
Khan S	17	M	0	3
SV	13	F	1	3
SA	12	F	1.5	2.5
BA	16	F	1	0
KL	17	M	1	0
PD	15	M	2	2
PR	15	M	0	1
DV	14	M	1	1
Bilquis	15	F	2	1
Tasnim	13	F	0	1.5

[a] Positive > 3 mm = 9/20 (45%).

TABLE 11. *Assessment of lepromin test by a "blind" observer.*[a]

Name	Age	Sex	Our reading	Reading by "blind" observer
PV	14	M	5	7
BA	16	F	0	0
KS	13	F	3	4
Khan L	17	M	0	0
Khan S	17	M	3	4
PD	15	M	2	2
AS	14	F	5	5
SV	15	F	5	7
SS	16	F	5	5
Bilquis S	15	F	2	1
Tasnim	13	F	4	2
DK	17	F	5	6

[a] The readings were taken at two-day intervals.

In this study a vaccine containing killed ICRC bacilli has been able to induce a persistent lepromin conversion in LL patients, associated, in some cases, with changes in tissue response consistent with up-grading of the lesions ([3, 11]). "Reactions" posed no problems and were easily controlled. Interestingly no permanent fresh nerve lesions were seen in spite of up-grading of immunity. Convit, *et al.* ([8]) have made similar observations.

Lepromin negativity has been somewhat higher than expected in the general population. However, similar results have been obtained by Castellani, *et al.* ([7]) using the same dose and source of the antigen. Lepromin conversion was observed in 91% of the lepromin-negative contacts. On the other hand, controls (nonvaccinated) did not exhibit any change in lepromin reactivity, indicating that the conversion was not merely a consequence of the previous lepromin test. In Malwani, the patients are not segregated, and thus even the noncontacts (general population) are frequently exposed to *M. leprae*. This may explain why no conversion was seen as the result of the first lepromin test in the controls. Reduction of the dose of the vaccine not only induced conversion in a lesser number of subjects but the magnitude of the response was also lower.

Convit, *et al.* ([10]) have shown that clearance of *M. leprae* is markedly retarded in LL patients; bacilli could be demonstrated in the skin at the site of injection even 30 days later. This may explain our observation of the development of induration at the site of the first lepromin test following vaccination in some patients and contacts. Thus three weeks after vaccination, a time required for sensitization of appropriate clones, the antigen would still be present at the site of the first test for interaction with sensitized lymphocytes.

About 40% of the LL patients in this study could not be converted to lepromin positivity. There was no correlation between conversion rates and host parameters such as age, bacillary load, and duration of treatment. This points to the existence of a subgroup within LL that would not respond to vaccine. Convit, *et al.* ([8]) have found a similar group even after repeated administration of their vaccine. Association of immune regulatory (Ir) genes with H-2 complex in mice is well established ([34]). HLA in man may likewise contain Ir genes that could control the immune response to *M. leprae*. Genetic mechanisms have been incriminated in the pathogenesis of leprosy ([21]). It would be interesting to investigate the genetic make up, including HLA typing, of the "nonresponders" to our vaccine to find out if they represent a genetically distinct group.

Although the CMI defect in leprosy is highly specific, its mechanism is still not well understood ([5, 27, 36]). Macrophage function has been found to be defective by some ([2]), although this has not been universally accepted ([13, 29]). A reduction in the number of T cells has been reported by a number of workers ([14, 25]). According to Godal, *et al.* ([19]) the main defect is "central failure" due to lack of specifically reacting cells. ICRC appears to be stronger antigenically as compared to *M. leprae*. Thus, in vaccinated LL patients, response to ICRCin was always stronger. Moreover, an equally good response was also seen even in those who could not be converted. In a separate study the two antigens administered simultaneously induced a comparable biphasic skin response. However ICRCin always induced larger induration than lepromin. The ICRC bacillus may thus provide strong crossreacting antigens which could activate appropriate clones breaking the "tolerance." Alternatively, the vaccine could act by enhancing the helper cells, or by suppressing the suppressor cells. Although several workers have demonstrated generation of suppressor cells during the course of infection by *M. lepraemurium* in mice ([6, 37]), there is no unanimity of opinion on the status of suppressor cells in LL patients ([24, 26]).

The data of this study, both in regard to patients and healthy contacts, strongly indicate immunogenic potentials of the ICRC vaccine. However, before initiating field trials it would be essential to get information on: 1) safety and acceptability, 2) optimal dose, 3) frequency of vaccination, 4) age of vaccination, and 5) antigenic stability of the organism.

Acute and chronic toxicity studies carried out in a large number of animals of different species show that the ICRC vaccine is nontoxic even when given in very high doses.

Likewise, no harmful effects have been seen in patients or in healthy volunteers ([11]). The reaction produced in LL patients is easily controlled. Being given as a single shot, the vaccine has high acceptability.

No differences have been observed in responses when the vaccine is given in a dose range of 27 μg–67 μg. The proposed dose in patients, for the future, will be 50 μg/patient (equivalent to 1×10^9 bacilli). At 50% of this dose, 90% of lepromin-negative contacts exhibited conversion. When the vaccine was given in very low dose (1.6×10^7 bacilli/person), conversion was observed in only 45%. As per the present estimate, the optimal dose in healthy individuals would be 1×10^8 bacilli/person.

Frequency of vaccine administration will depend upon the period for which lepromin conversion is observed. Lepromin conversion has been persistent in patients even after three years. Similar studies with reference to contacts are in progress.

The immune system is relatively immature in the newborn ([34]), and no useful purpose would be served by giving the vaccine at a time when the body has not yet developed the power to respond to antigens of *M. leprae*. This is an important point because immature lymphocytes exposed to excess antigen, as would be the case after vaccination, may in fact develop immunological tolerance. In our studies very low lepromin positivity was observed below ten years of age, and similar results have been obtained by Guinto, *et al.* ([20]). In their studies, 90% of even the household contacts exhibited a negative lepromin reaction below the age of five years. In view of the data, we have decided to give the vaccine only to children above five years of age.

ICRC bacilli (now in the 89th passage) appear to be antigenically stable, at least with reference to the crossreacting CMI antigens. All vaccine batches used so far have produced good lepromin conversion in LL patients. Likewise, when the vaccine was given in antigenic doses, the magnitude of the Mitsuda response was similar with different batches in TT patients.

A number of effective drugs which reduce bacillary load in patients are now readily available. Since man is practically the only reservoir for leprosy germs, it should be theoretically possible to wipe out the disease by drug therapy. However, drug resistance is occurring at an alarmingly high rate ([31]), and there is an urgent need to develop a vaccine against leprosy. The data obtained so far make the ICRC vaccine a highly promising candidate vaccine which should soon undergo field trials.

Acknowledgments. The authors are grateful to Dr. C. K. Deshpande, Dean, G. S. Medical College, for his permission and help in conducting studies at the community center in Malwani, Bombay. Grateful acknowledgment is made for the valuable help and advice rendered by Dr. Vidya Acharya, Professor and Chairman, Department of Medicine, G. S. Medical College. Grateful thanks are due to the management of the Acworth Leprosy Hospital, Wadala, for extending facilities for work on patients. The skillful technical assistance given by Mr. A. V. Bhat, Cancer Research Institute, is especially acknowledged.

The vaccine was manufactured under licence No. 1435 and No. 1594 from the Food and Drug Administration, Maharashtra State, under the advice and clearance of the Drug Controller, India. The trials were cleared by our Ethical Committee, and free and informed consent of the subjects was obtained.

REFERENCES

1. Bapat, C. V., Randive, K. J. and Khanolkar, V. R. *In vitro* cultivation of an acid fast mycobacterium isolated from human lepromatous leprosy. Indian J. Pathol. Bacteriol. **1** (1958) 156–159.
2. Beiguelman, B. Leprosy and genetics. A review of past research with remarks concerning future investigations. Bull. WHO **37** (1967) 461–476.
3. Bhatki, W. S., Chulawala, R. G., Bapat, C. V. and Deo, M. G. Reversal reaction in lepromatous patients induced by a vaccine containing killed ICRC bacilli—a report of five cases. Int. J. Lepr. **51** (1983) 466–472.
4. Bjune, G., Barneston, R. St. C., Ridley, D. S. and Kronvall, G. Lymphocyte transformation test in leprosy; correlation of the response with inflammation of lesions. Clin. Exp. Immunol. **25** (1976) 85–94.
5. Bullock, W. E. Immunology and the therapeutics of leprosy. Ann. Intern. Med. **91** (1979) 482–483.
6. Bullock, W. E., Carsen, E. M. and Gershon, R. K. The evolution of immunosuppressive cell populations in experimental mycobacterial infection. J. Immunol. **120** (1978) 1709–1716.
7. Castellani, G. T., Castellani, G. L. T., Dinle, Y. H., Mohamed, F. A., Yacub, A. H., Agib, A. M. and Mahamud, I. A. Investigaciones sobre la cicatriz post-leprominica. Rev. Fontilles **13** (1981) 155–190.
8. Convit, J., Aranzazu, N., Alvarado, M., Zuniga, M., Ulrich, M., Aragon, M. E., Reyes, O. and

TELLEZ, S. Immunotherapy and immunoprophylaxis of leprosy. Proc. III Mtg. Standing Committee of Leprosy Control in Caribbean, Santo Domingo, Dominican Republic, 7–9 June, 1982, 123–134.

9. CONVIT, J., ARANZAZU, N., PINARDI, M. E. and ULRICH, M. Immunological changes observed in indeterminate and lepromatous leprosy patients and Mitsuda-negative contacts after inoculation of a mixture of *Mycobacterium leprae* and BCG. Clin. Exp. Immunol. **36** (1979) 214–220.
10. CONVIT, J., ULRICH, M. and ARANZAZU, N. Vaccination in leprosy—observations and interpretations. Int. J. Lepr. **48** (1980) 62–65.
11. DEO, M. G., BAPAT, C. V., BHATKI, W. S. and CHULAWALA, R. G. Potential anti-leprosy vaccine from killed ICRC bacilli—a clinicopathological study. Indian J. Med. Res. **74** (1981) 164–177.
12. DHARMENDRA and CHATTERJEE, K. R. Prognostic value of the lepromin test in contacts of leprosy cases. Int. J. Lepr. **24** (1956) 315–318.
13. DRUTZ, D. J. and CLINE, M. J. Polymorphonuclear leukocyte and macrophage function in leprosy. Int. J. Lepr. **38** (1970) 352–353.
14. DWYER, J. M., BULLOCK, W. F. and FIELDS, J. P. Disturbance of blood T:B lymphocyte ratio in lepromatous leprosy. Clinical and immunological correlations. N. Engl. J. Med. **188** (1973) 1036–1039.
15. FABER, W. R., LEIKER, D. L., NENGERMAN, I. M., ZEIJLEMAKER, W. P. and SCHELLEKENS, P. T. A. Lymphocyte transformation test in leprosy: Decreased lymphocyte reactivity to *Mycobacterium leprae* in lepromatous leprosy, with no evidence for a generalized impairment. Infect. Immun. **22** (1978) 649–656.
16. GANGAL, S. G. and KHANOLKAR, S. R. Delayed hypersensitivity *in vitro* to an acid fast mycobacterium cultivated from human lepromatous leprosy. Indian J. Med. Res. **62** (1974) 290–296.
17. GIRDHAR, B. K. and DESIKAN, K. V. Results of skin tests with five different mycobacteria. Lepr. India **50** (1978) 555–559.
18. GODAL, T. Is immunoprophylaxis in leprosy feasible? [The Clayton memorial lecture, 1978.] Lepr. Rev. **48** (1978) 305–317.
19. GODAL, T., MYRVANG, B., FROLAND, S. S., SHAO, J. and MELAKU, G. Evidence that the mechanism of immunological tolerance ("central failure") is operative in the lack of host resistance in lepromatous leprosy. Scand. J. Immunol. **1** (1972) 311–321.
20. GUINTO, R. S., DOULL, J. A. and MABALAY, E. B. The Mitsuda reaction in persons with and without household exposure to leprosy. Int. J. Lepr. **23** (1955) 135–138.
21. HARBOE, M. Genetic aspects of leprosy. In: *Modern Genetic Concepts and Techniques in the Study of Parasites*. F. Michal, ed. WHO Tech. Rep. Ser. No. 4, 1981, pp. 387–409.
22. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo (*Dasypus novemcinctus*, Linn.) as a model for the study of leprosy. 1. Report of lepromatoid leprosy in an experimentally infected armadillo. Int. J. Lepr. **39** (1971) 693–702.
23. MACKANESS, G. B. The relationship of delayed hypersensitivity to acquired cellular resistance. Br. Med. Bull. **23** (1967) 52–54.
24. MEHRA, V., MANSON, L. H., FIELDS, J. P. and BLOOM, B. R. Lepromin-induced suppressor cells in patients with leprosy. J. Immunol. **123** (1979) 1813–1817.
25. NATH, I., CURTIS, J., BHUTANI, L. K. and TALWAR, G. P. Reduction of a sub-population of T lymphocytes in lepromatous leprosy. Clin. Exp. Immunol. **8** (1974) 81–87.
26. NATH, I. and SINGH, R. The suppressive effect of *M. leprae* on the *in vitro* proliferative responses of lymphocytes from patients with leprosy. Clin. Exp. Immunol. **41** (1980) 406–414.
27. NAVALKAR, R. G. Immunology of leprosy. CRC Crit. Rev. Microbiol. **8** (1980) 25–47.
28. NEWELL, K. W. An epidemiologist's view of leprosy. Bull. WHO **37** (1967) 461–476.
29. PARMESWARAN, M., GIRDHAR, B. K., DEO, M. G., KANDHARI, K. C. and BHUTHANI, L. K. Macrophage function in leprosy. Int. J. Lepr. **44** (1976) 340–345.
30. REES, R. J. W. The significance of the lepromin reaction in man. Prog. Allergy **8** (1964) 224–258.
31. REES, R. J. W. An appraisal of medical research in the treatment and control of leprosy. J. R. Soc. Arts **130** (1982) 186–198.
32. REES, R. J. W., WATERS, M. F. R., PEARSON, J. M. H., HELMY, H. M. and LAING, A. B. G. Long-term treatment of dapsone-resistant leprosy with rifampicin: Clinical and bacteriological studies. Int. J. Lepr. **44** (1976) 159–169.
33. RIDLEY, D. S. and JOPLING, W. H. Classification of leprosy according to immunity. A five group system. Int. J. Lepr. **34** (1966) 255–273.
34. ROITT, I. M. *Essential Immunology*. 3rd ed. London: Blackwell Scientific Publications, 1977.
35. SANSARRICQ, H. Recent advances and present trends in leprosy. Experientia **33** (1977) 114–119.
36. TURK, J. L. and WATERS, M. F. R. Cell-mediated immunity in patients with leprosy. Lancet **2** (1969) 243–246.
37. TURCOTTE, R. Suppressor cells in experimental murine leprosy. Int. J. Lepr. **46** (1979) 358–363.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Two Candidate Antileprosy Vaccines—Current Status of Their Development[1]

Gursaran P. Talwar and Arun Fotedar[2]

Epidemiological data from endemic areas indicate that a very large majority (over 95%) of subjects are able to resist infection and do not develop leprosy. An eventual vaccine should therefore have the ability to invigorate immunity in that segment of the population which falls prey to this disease and, in particular, in those who develop lepromatous leprosy (LL).

Lepromatous leprosy patients have a long-lasting defect for response to *Mycobacterium leprae* antigens, as measured by depressed blast transformation of leukocytes and the inability to generate lymphokines influencing monocyte migration. These patients give negative lepromin reactions ([2]), and macrophages from such patients are unable to eliminate the bacilli.

The basis of the immunological deficit in lepromatous leprosy patients is only partially understood. Investigations carried out by de Vries, *et al.* ([1]) failed to show a distinct correlation of HLA loci with lepromatous leprosy. If at all, the association was with tuberculoid leprosy. The role of suppressor cells in the immunological defect in lepromatous leprosy is not clear. Some workers demonstrate increased activity of *M. leprae*-generated T suppressor cells in lepromatous leprosy; whereas others find enhanced T suppressor cells in tuberculoid leprosy. Whatever be the mechanism, it is evident that tolerance, or anergy of response to *M. leprae* antigens, exists in these patients. An eventual antileprosy vaccine should therefore be effective in breaking this state of immunological tolerance and in stimulating the deficient immune responses in actual (and potential) lepromatous leprosy patients.

Two candidate vaccines are proposed: 1) a vaccine based on an atypical cultivable mycobacteria which resembles *M. leprae* in several cell-mediated immune reactions with cells of tuberculoid leprosy patients, but is also partially different, and 2) vaccine based on hapten-modified *M. leprae.*

The rationale in both cases is to break the tolerance of response to *M. leprae* antigens.

Characteristics and properties of the proposed vaccines

Derivatized *M. leprae.* Acetoacetyl groups have been used as haptens to shift a predominantly humoral response to a dominant cell-mediated immune response against a number of antigens ([5]). Since lepromatous leprosy is characterized by depressed, cell-mediated immune responses, acetoacetylation of *M. leprae* was carried out to prepare the derivative with modified properties. Irradiated *M. leprae* was purified from biopsies by a procedure which gives negligible tissue contamination on microscopic examination. Essentially, the procedure consisted of homogenization of biopsy material in distilled water, followed by collagenase and trypsin treatments, and separation of bacilli on a discontinuous sucrose gradient (5:10:40). The bacilli were derivatized with diketene freshly prepared in the laboratory. The other product of reaction, e.g., acetoacetic acid, was washed off during the preparation procedure. The preparation was sterile as checked on thioglycolate, chocolate agar, and nutrient agar. It was nonpyrogenic in rabbits and nontoxic in

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] G. P. Talwar, D.Sc., F.I.C.A., F.A.Sc., F.N.A.; A. Fotedar, M.B., B.S., ICMR-WHO Research and Training Centre in Immunology, Department of Biochemistry, All-India Institute of Medical Sciences, New Delhi 29, India.

standard acute toxicity studies in mice and guinea pigs.

Acetoacetylated *M. leprae* gave significantly higher inhibition of leukocyte migration when tested with cells of lepromatous leprosy patients, suggesting the ability of this antigen to improve the production of lymphokines as compared to nonmodified *M. leprae* ([3]). Immunization of mice with acetoacetylated *M. leprae* confers on them protective immunity against challenge with viable *M. leprae* in the foot pad.

Mycobacterium w. *Mycobacterium w* was selected from a panel of 15 cultivable mycobacteria because of its antigenic relatedness to *M. leprae*, as assayed by the blast transformation and leukocyte migration inhibition (LMI) assays with leukocytes of tuberculoid leprosy patients ([4]). The skin reactions with homologous and heterologous antigens in sensitized guinea pigs and the analogous delayed-type hypersensitivity skin responses in tuberculoid leprosy patients further suggested the antigenic relatedness of *Mycobacterium w* with *M. leprae*. In lepromatous leprosy patients *Mycobacterium w* elicited reactions; whereas *M. leprae* did not. *Mycobacterium w* is a rapid grower and on the basis of metabolic characteristics is classifiable in Runyon's Group IV ([6]). *Mycobacterium w* causes the enlargement of draining lymph nodes but was found to be nonprotective in the mouse foot pad system ([7] and unpublished data).

Protocol of preliminary clinical studies

With the approval of the appropriate authorities, probing field trials on a limited number of subjects have been carried out with these vaccines to see if lepromin negative subjects could be converted to lepromin positivity status.

The test subjects were: a) contacts and relations of lepromatous leprosy patients, and b) borderline lepromatous (BL) and lepromatous leprosy patients rendered bacteriologically negative by chemotherapy.

In both cases, initial lepromin testing was carried out with Dharmendra and Mitsuda lepromins, and only those subjects who were negative to lepromin on repeated testing were taken for study. The immunization was carried out with the candidate vaccines, injecting intradermally 5×10^7 bacilli suspended in 0.1 ml normal saline. Lepromin retesting was done 4–6 weeks after immunization.

RESULTS

Studies in BL/LL patients. So far, 32 patients have been studied at the School of Tropical Medicine, Calcutta, India (Dr. S. Chaudhri) and five patients at the Hemerijckx Leprosy Centre, Polambakkam (Dr. C. Vellut and Dr. Maroof Sahib).

In the Calcutta study, 32 patients were tested. They were repeatedly lepromin negative (four-week Mitsuda lepromin reading was less than 3 mm and the 48-hr Dharmendra lepromin response was less than 10 mm). Out of these patients, 20 showed conversion to lepromin positivity status after a single intradermal injection of *Mycobacterium w*. The response of the converted subjects with Mitsuda lepromin was between 5 mm–12 mm induration at four weeks (negative reading was <3 mm), and with Dharmendra lepromin the response was 10 mm–20 mm erythema at 48 hr. Biopsies were taken from 12 of those converted to lepromin positivity status, and the histology showed massive mononuclear infiltration in all biopsies of positive Mitsuda lepromin reactions. In some cases granuloma formation could be seen, and giant cells were seen in three cases.

Studies in lepromin negative contacts. In all, 30 repeatedly lepromin negative contacts were studied at Calcutta. They were immunized with *Mycobacterium w*; 28 out of 30 became positive to the Mitsuda test and 30 out of 30 to the Dharmendra lepromin test.

Studies on contacts were also carried out at Behrampur Aska (Drs. Sahu, Jena, Mullick, Singh, and Sengupta). Ten out of 13 contacts converted to positivity after immunization with *Mycobacterium w*, and 7 out of 13 converted to lepromin positivity after immunization with acetoacetylated *M. leprae*. The histopathology of the converted cases showed massive mononuclear infiltration.

In another study carried out at Behrampur, the following protocol was used: 14 repeatedly lepromin negative contacts were immunized with autoclaved *M. leprae* at a dose of 5×10^7 bacilli in 0.1 ml saline intradermally. On retest, two converted to lepromin positivity. The remaining 12 sub-

jects were immunized with 5×10^7 autoclaved *Mycobacterium w* intradermally. Seven out of nine immunized with *Mycobacterium w* were reported converted to lepromin positivity status. Three were lost to follow up.

A pertinent question that arises is whether the delayed hypersensitivity (DTH) skin response evoked to *M. leprae* antigens in BL/LL patients on immunization with *Mycobacterium w* vaccine is transient or has a lasting duration. It can also be asked whether the apparent improvement in immune response as assessed by DTH is matched by any other criteria of cell-mediated immunity. Recently Dr. Fotedar from the All-India Institute of Medical Sciences (AIIMS) laboratory visited Calcutta to retest the subjects immunized with *Mycobacterium w*-based vaccine 9–10 months previously. Leprosin A (obtained from WHO-IMMLEP through the Indian Council of Medical Research) was used for skin tests. To evaluate the ability of peripheral cells to produce lymphokines in the presence of *M. leprae* antigens, LMI assays were also carried out. Subjects retested belonged to both categories—those who had converted to lepromin positivity, and those who failed to do so and remained lepromin negative after a single intradermal injection of 5×10^7 of killed *Mycobacterium w*. The identity of the converted and nonconverted cases was not revealed to Dr. Fotedar at the time of the retest, but his results confirmed correctly the converted cases. These cases again gave a positive skin response to leprosin A, and their LMI index was less than 61. Among the previous three negatives retested in the second batch, all failed to produce leukocyte migration inhibition. Two were also negative to leprosin A in skin response; the third, however, gave a positive skin test. This case in an earlier test was negative to Mitsuda lepromin but had a 10 mm erythema at 48 hr with Dharmendra lepromin.

These preliminary studies, limited to 37 BL/LL patients and 56 contacts, indicate the potential of *Mycobacterium w* and acetoacetylated *M. leprae* to convert lepromin negative subjects to lepromin positivity status. Retesting of *Mycobacterium w*-vaccinated cases showed the stable nature of the change for about nine months after the initial vaccination.

REFERENCES

1. DE VRIES, R. R. P., LAI-A-FAT, R. F. M., NIJENHUIS, L. E. and VAN ROOD, J. J. HLA-linked genetic control of host response to *Mycobacterium leprae*. Lancet **2** (1976) 1328–1330.
2. DHARMENDRA. *The Lepromin Reaction*. London: British Empire Leprosy Relief Association, 1948.
3. FOTEDAR, A., MUSTAFA, A. S., NARANG, B. S. and TALWAR, G. P. Improved leucocyte migration inhibition response of leucocytes from lepromatous leprosy patients with hapten modified *M. leprae*. Clin. Exp. Immunol. **49** (1982) 317–324.
4. MUSTAFA, A. S. and TALWAR, G. P. Five cultivable mycobacterial strains giving blast transformation and leukocyte migration inhibition of leukocytes analogous to *Mycobacterium leprae*. Lepr. India **50** (1978) 498–508.
5. PARISH, C. R. The relationship between humoral and cell-mediated immunity. Transplant. Rev. **13** (1972) 35–66.
6. SAXENA, V. K., SINGH, U. S. and SINGH, A. K. Bacteriological study of a rapidly growing strain of *Mycobacterium*. Lepr. India **50** (1978) 588–596.
7. SHEPARD, C. C., VAN LANDINGHAM, R. and WALKER, L. L. Searches among mycobacterial cultures for antileprosy vaccines. Infect. Immun. **29** (1980) 1034–1039.

INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Natural History of Leprosy—Aspects Relevant to a Leprosy Vaccine[1]

Paul E. M. Fine[2]

The population pattern of leprosy is reasonably well known but poorly understood. One major problem is that current epidemiological information relates almost entirely to clinical disease; whereas the underlying pattern of infection remains a matter of conjecture. The relationship between infection and disease has important implications for leprosy control either by drugs or by vaccine. Another major problem area concerns the mechanisms which underlie host response to infection and which determine disease type. Given that these mechanisms are probably largely immunological, a sound understanding of their nature is necessary for rational design and application of immunoprophylactic tools.

Patterns in populations—clinical leprosy

Leprosy is widespread but neither stable nor homogeneous ([5, 6]). Dramatic changes in prevalence and incidence have been observed over periods of years (Nauru), decades (Norway) or centuries (Europe). The capacity for such changes in the absence of disease control means a necessity for extreme caution when interpreting trends in a trial or control program. Appropriate comparison populations are essential. Another general feature of leprosy is its tendency to cluster in regions, villages and households. Insofar as factors (genetic, exposure, intercurrent infection) responsible for clustering may influence the action of a vaccine, the phenomenon is of relevance of vaccine trials, and it implies that randomization should be on an individual rather than group basis. Major differences in clinical type distribution are reported for different regions of the world, e.g., a low proportion of lepromatous cases in Africa compared to Asia or South America. It is important to assess to what extent these differences are attributable to regional variation in case ascertainment and diagnostic practices, since they have major implications for the distribution of sources of infection in the community and for the response of different populations to infection. Insofar as populations which respond differently to natural infection may respond differently to a vaccine, these patterns are important for the design and interpretation of a vaccine trial.

Leprosy is an uncommon disease, even in highly endemic areas. Few populations have prevalence rates in excess of 0.02 or incidence rates in excess of 0.001 per year, and incidence rates of lepromatous disease may be an order of magnitude lower than this. Such low rates mean that large populations must be followed for many years in a vaccine trial. Indeed, no trial has yet included sufficient lepromatous cases to assess whether BCG is effective against multibacillary disease. Incidence rates generally rise to a peak in the 10–25 year age group; thus a concentration on young adults may increase the efficiency of a trial design. Trial size might be further reduced by concentrating upon household contacts, since it is known that contacts of lepromatous cases have an approximately tenfold risk of disease, and contacts of tuberculoid cases have an approximately twofold risk of disease compared to noncontacts. But such an approach has major disadvantages. There are logistic difficulties in identifying and following up a specially selected population spread out over a large area. In addition, household contacts are not representative of general

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] P. E. M. Fine, V.M.D., Ph.D., Ross Institute, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, England.

populations for reasons of their genetic similarity to cases and high levels of exposure. Insofar as a) these factors might be relevant for the action of vaccine, and b) most incident cases in endemic populations appear in individuals not exposed in their household, the results of such a trial might be inappropriate for decisions concerning general populations.

Transmission of *Mycobacterium leprae*

Much of the literature on the epidemiology of leprosy concentrates upon transmission, as though this were the key to its natural history ([5]). A human reservoir is generally presumed—it is ironic that the only known animal reservoir, New World armadillos, should represent a reverse zoonosis in an area into which leprosy was introduced by man. Recent years have witnessed a return to the old view that transmission is largely airborne, from the upper respiratory tract of multibacillary cases. The portal of entry remains unknown but may include both skin and the mucosal lining of the respiratory tract ([2]). Other mechanisms are repeatedly discussed—arthropods, breast milk, transplacental—but there is no evidence that they play a major role.

We may ask ourselves how relevant is the issue of transmission for a leprosy vaccine. It may be argued that transmission has been over-emphasized, and that the distribution of clinical leprosy is due more to factors determining the response to established infection than to risk factors for infection itself. On the other hand, it is important for leprosy control purposes to identify the sources of *M. leprae* transmission in a community and to resolve the controversy of whether or not transmission is due entirely to multibacillary cases. A vaccine which did not prove effective against the sources of infection might, in principle, lower morbidity but not reduce the risk of infection in the community.

What determines clinical type?

Just why one individual manifests tuberculoid and another lepromatous disease remains one of the major outstanding puzzles of leprosy. Whatever the determining factor(s) may be, it is (they are) present everywhere since the clinical spectrum is a universal feature of leprosy. Six mechanisms have been suggested: 1) Variation in *M. leprae*—but there is no evidence for a correlation between any bacterial characteristic and either geographic origin or clinical type of the source case. 2) Host genetics—although there is evidence for some influence of an HLA-linked factor in tuberculoid disease, the genetics of the lepromatous response has not been well studied due to the rarity of multiple lepromatous cases in single families. Indeed, the fact that type concordance rates within families are not particularly high is one argument against a strong genetic influence. At the least it implies that such influence must involve several genes ([3]). 3) Dose or superinfection—it has been argued that a large inoculum might predispose to lepromatous disease ([4, 6]). Such a mechanism should lead to high risk of lepromatous disease among contacts of multibacillary cases, but there is very little evidence for this. 4) Route of infection—the possibility that antigen presentation may control immunological response, in particular the possibility that a primary infection of dermal Langerhans' cells may elicit strong cell-mediated reactions, is currently an area of exciting research. 5) Physiological state—although it has been postulated that conditions associated with depressed cell-mediated immunity, such as pregnancy or protein deficiency, determine immune responses in leprosy, convincing evidence is still lacking. 6) Immunological background—the fact that BCG has been protective in three major trials, and leprosy incidence was correlated inversely with initial tuberculin sensitivity in Uganda, suggest that host experience with different mycobacteria may determine their response to *M. leprae* infection ([8]).

It is frequently assumed that a useful antileprosy vaccine should protect against multibacillary disease. It is thus important to know to what extent the determining factors are immunologically manipulable.

Subclinical infection

One of the problems in discussing subclinical infections with *M. leprae* is semantic—does the term include preclinical (incubating) infections, persistent infection without clinical manifestation, and/or sterile self-cure? Each may occur, but each involves a very different mechanism. Several

lines of evidence now suggest that the incidence of *M. leprae* infection may be far greater than the incidence of clinical leprosy: 1) presence of *M. leprae*-specific antibodies among large numbers of normal persons in an area of low leprosy endemicity ([1]); 2) evidence for sensitized lymphocytes among contacts ([4]); 3) skin test sensitivity among contacts as elicited by soluble *M. leprae* antigens; 4) presence of *M. leprae*-like bacilli in skin of clinically normal contacts; 5) analogies with tuberculosis, in which only 5% of infected persons may suffer clinical illness; 6) recognition that many leprosy lesions heal completely of their own accord, and thus many mild cases will never be recognized; and 7) theoretical extension of the spectrum beyond mild self-healing types to forms which never manifest clinically at all.

Knowledge of the prevalence and distribution of infection is important for vaccine studies insofar as a vaccine might protect only if given before initial infection occurs. Such a restriction is consistent with the finding that BCG was protective only if given before five years of age in Burma. An ideal vaccine trial should be capable of assessing the relationship of prior exposure and infection to vaccine efficacy.

Atypical mycobacteria

The relevance of environmental mycobacteria has been much discussed as a possible explanation for the different results of BCG trials against tuberculosis. Similar variations in results of BCG trials against leprosy suggest the extension of these arguments to leprosy as well. Laboratory experiments have shown that different mycobacterial infections affect the efficacy of BCG protection against tuberculosis in animals. It might even be argued that BCG is itself an "atypical" mycobacterium, in that it is an artificial species dependent upon laboratory media for its persistence in nature and sharing several antigens with most mycobacteria, including *M. leprae* and *M. tuberculosis*. One of the chief difficulties in studies of the interactions between mycobacteria is the great number and variety of species throughout the world ([7]).

The prospect of unravelling the implications of different mycobacteria is daunting in complexity, but there is ample evidence that these interactions are important and should not be overlooked. Indeed, perhaps the basic question to be addressed in a leprosy vaccine trial should not be "Does the vaccine protect?" but "Why does protection vary?"

REFERENCES

1. Abe, M., Minagawa, F., Yoshino, Y., Ozawa, T., Saikawa, K. and Saito, T. Fluorescent antibody absorption (FLA-ABS) test for detecting subclinical infection with *Mycobacterium leprae*. Int. J. Lepr. **48** (1980) 109–119.
2. Davey, T. F. The nose in leprosy: Steps to a better understanding. Lepr. Rev. **45** (1974) 97–103.
3. Fine, P. E. M. Immunogenetics of susceptibility to leprosy, tuberculosis and leishmaniasis: An epidemiological perspective. Int. J. Lepr. **49** (1981) 437–454.
4. Godal, T. Immunological aspects of leprosy—present status. Prog. Allergy **25** (1978) 211–242.
5. Guinto, R. S. Epidemiology of leprosy: Current views, concepts and problems. In: *A Window on Leprosy*. Chatterjee, B. R., ed. Ghandi Memorial Leprosy Foundation, 1978, pp. 36–53.
6. Irgens, L. M. Leprosy in Norway—an epidemiological study based on a national patient registry. Lepr. Rev. **51** Suppl. (1980) 1–130.
7. Rook, G. A. W., Bahr, G. M. and Stanford, J. L. The effect of two distinct forms of cell-mediated response to mycobacteria on the protective efficacy of BCG. Tubercle **62** (1981) 63–68.
8. Stanley, S. J., Howland, C., Stone, M. M. and Sutherland, I. BCG vaccination of children against leprosy in Uganda: Final results. J. Hyg. (Lond.) **87** (1981) 233–248.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Epidemiological Considerations in Vaccine Trials in Leprosy[1]

S. K. Noordeen[2]

Development of an antileprosy vaccine in order to achieve control of leprosy through primary prevention is a major objective of the Immunology of Leprosy (IMMLEP) Scientific Working Group, a component of the UNDP/World Bank/WHO Special Programme on Research and Training in Tropical Diseases. The progress made so far in the development of the vaccine and the results from animal experimentation are very promising. However, in the ultimate analysis, no vaccine can be used in control programs unless it has been tested and found satisfactory in human subjects. While evaluation of side-effects, acceptance, and immune response could be studied in small groups of human subjects, the evaluation of a vaccine in terms of its protective capability in the individual and its capacity to bring about disease control in the community, can be effected only through large-scale prospective studies in populations. There appears to be no viable alternative to this approach.

Before going into the epidemiological issues, it is necessary to consider the kinds of vaccine testing in man that are relevant to the measurement of the efficacy of a leprosy vaccine. These, not necessarily in order of sequence, are: 1) measuring immune conversion in a) healthy uninfected subjects through an immunological test, and b) leprosy patients who are CMI deficient; 2) measuring protection in high-risk groups (e.g., contacts); and 3) measuring protection in total populations as a public health experiment. While the first two steps are very important in establishing the immune potential of the vaccine in man, measurement of the public health impact of the leprosy vaccine will be possible only through the third step.

A large-scale field trial with a leprosy vaccine could answer one or more of the following questions:

1) To what extent does the vaccine protect against leprosy of multibacillary types? paucibacillary types?

2) To what extent does the vaccine protect the uninfected population? the infected population?

3) What is the duration of protection against different types? among the different populations?

4) To what extent does the vaccine cure leprosy in lepromatous leprosy? borderline leprosy? indeterminate leprosy?

5) To what extent does the vaccine produce side-effects in the uninfected population? the infected population? the diseased population?

6) To what extent does the absence of local reaction to vaccine and/or negative skin test reactivity after vaccination predict lepromatous leprosy?

In a public health experiment of the vaccine involving a total population, the protection measured will depend upon a) the composition of the population (The Table) and b) the effect of the vaccine on specific categories of the population.

The most successful vaccine in leprosy would be one which would prevent disease in both uninfected and infected populations. This would certainly bring about early control or even eradication of the disease. On the other hand, if the vaccine is effective only among the uninfected then it will take a long time to control leprosy in high en-

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] S. K. Noordeen, M.B., B.S., D.P.H., M.P.H. (Michigan), Secretary, IMMLEP and THELEP Steering Committees, Leprosy Unit, WHO Headquarters, Geneva, Switzerland.

THE TABLE. *Composition of the population.*

Uninfected	Infected	Diseased
unexposed	not incubating	minimal early
exposed	incubating	established
		paucibacillary
		multibacillary

demic areas, since the population will have only a low proportion of uninfected individuals. In low endemic areas the disease could be controlled earlier because of a high proportion of uninfected individuals. However, in view of the low-risk situation, the number of persons to be vaccinated in order to prevent one case of leprosy will be quite large, making a vaccine strategy comparatively less attractive from the cost-benefit point of view.

In light of the above, the epidemiological considerations for a large-scale vaccine trial will depend upon the following:

Composition of the population. The composition of the population could vary in terms of proportions which could be identified as: a) uninfected, b) infected but with adequate cell-mediated immunity (CMI) against *Mycobacterium leprae,* c) infected but with inadequate CMI against *M. leprae,* and d) diseased with varying degrees of CMI.

It is possible that the degree of efficacy of the vaccine could be different in the different groups. The composition itself will depend upon the prevalence and incidence of disease as well as infection in the population, and on the proportion of individuals who are possibly incapable of mounting a CMI response.

Expected incidence of leprosy. An important epidemiological consideration for vaccine trials would be the expected incidence of leprosy and of its various types in the control group of the study population. Unless the yield of new cases in the control group is adequate, comparison with the vaccinated group for evaluating the degree of protection may not be possible. The expectations on incidence can only be based on past experience in the population. However, the very presence of a research team and possible intensification of control activities, including improved chemotherapy, may contribute to reduction in incidence even in the control group, thus leading to the earlier expectations not being met. In order to compensate for such contingencies, provisions may have to be made for either extension of the population size or extension of the period of follow up, or both. There are inherent difficulties in both options. In any case, it is most prudent to work on minimal expectations of incidence.

Trial on high-risk groups. One of the options often proposed is to confine the trials to high-risk groups, such as contacts or specific age groups, and thus enable the size of the trial population to remain relatively small. With the complex potentialities of the leprosy vaccine, it is rather difficult to generalize the degree of efficacy observed in high-risk groups to other groups having a relatively low risk. Further, the currently identifiable high-risk groups yield only a minority of new cases in most leprosy endemic situations and, therefore, the impact of the vaccine as a public health measure will be quite limited if vaccination is confined to identifiable high-risk groups. Lastly, under program conditions, vaccination of selective groups is not likely to be cost effective.

BCG vaccination status. The status of the trial population with regard to BCG vaccination will be an important consideration in view of the existing information on the varying levels of protection against leprosy observed in different areas with BCG vaccination. The consideration will include the extent of coverage in different age groups and the period elapsed since vaccination. If the population had had a high BCG coverage, then comparison of the leprosy vaccine against a truly unvaccinated control will not be possible, and the comparison can be only between the leprosy vaccine plus BCG given earlier and BCG given earlier. This may be quite acceptable since, in the real-life situation, leprosy vaccine is likely to be used only in populations which had received BCG earlier as part of a tuberculosis control service.

Other mycobacterial infections. An epidemiological consideration of some consequence is the prevalence and incidence of tuberculous disease and infection and infections from environmental mycobacteria. The varying protection of BCG against tuberculosis and leprosy has often been at-

tributed, at least in part, to the prevalence of environmental mycobacterial infections. It is also possible that the prevalence and incidence of tuberculosis in the community may have some influence on leprosy and on the protective effect of the leprosy vaccine. Again, it is not advocated that one should look to a leprosy endemic population free from tuberculosis or environmental mycobacteria for the trial, but that one should be in a position to evaluate, if at all possible, any interference from these mycobacteria.

Diagnostic methods. The methods selected for measurement of the protective effect of the vaccine are vital to the final outcome. The ultimate end point for measurement will have to be the disease itself, in particular the types of disease which are progressive. However, it would be most valuable to measure immune conversion through intermediate indicators, provided that their specificity and sensitivity are adequate. Although we do not have such reliable and well-tested intermediate indicators as yet, the prospects are bright for having such tests available in the near future.

Measurement of the disease in the population, although it is most desirable to evaluate the vaccine against development of progressive forms such as lepromatous leprosy, may not always be possible since the disease is likely to be treated in the trial area even in its early minimal phase. Further, detection of early disease, even with histopathological support, is to an extent subjective. If sufficient precautions are not taken to ensure objective diagnosis of new cases occurring in the trial, there is a likelihood of over-estimation of new disease. Although one could argue that such overestimation will occur to the same extent in both the vaccinated and the control groups and thus is of no serious consequence, one should not overlook the fact that overdiagnosis will lead to a dilution of the numbers of true new cases in both the vaccinated and control groups, resulting in an underestimation of the protective effect of the vaccine. Conversely, any unduly stringent procedure which excludes possible new cases in the trial will not result in an over-estimation of the protective effect.

Other considerations. Apart from the epidemiological considerations already discussed, the outcome of a vaccine trial will also depend upon the type of trial proposed, whether totally randomized or a type of comparison between two areas; the follow-up procedures planned, whether continuous or interrupted; and the adequacy of the population size from the statistical point of view. Some of the factors can have significant influence on the over- and under-estimation of incidence and thus, indirectly, on the measurement of protection.

It is evident that no matter how promising a leprosy vaccine is in the laboratory and in small-scale studies, the ultimate answer to its value in the control of the disease can come only through large-scale field studies. The epidemiological considerations for such studies are many, the significance of all of which are not fully understood. Further, it may not always be possible for the population selected for study to meet all of the epidemiological requirements, and a degree of compromise in certain situations may become inevitable. Finally, the time horizon for vaccine trails, which may run into several years and as much as a decade, should be fully realized by everyone.

INTERNATIONAL JOURNAL OF LEPROSY Volume 51, Number 4
Printed in the U.S.A.

Report of the SEARO/IMMLEP/THELEP Joint Scientific Meeting on Leprosy, Rangoon, Burma, 18-19 November 1981, and the Joint Indian and IMMLEP Scientific Meeting on Immunoepidemiology of Leprosy, New Delhi, India, 14-16 February 1983.

Operational Problems in Vaccine Trials[1]

Johannes Guld[2]

The general principles of a control trial are now well understood. The trial shall have a design that is clear and explicitly formulated. The study population may be defined as all persons in a given area, or selected according to age or to (well-defined) risk. A control group is always to be included, e.g., one half of the study population or some other proportion. The allocation of the individual to the vaccinated group or the control group is to be strictly random, i.e., as decided by a throw of unloaded dice, or a well-shuffled pack of playing cards, or the use of published tables of random numbers. To the extent possible, the study should be blind, i.e., the individual participant should not know whether he has been vaccinated or not, and double-blind, i.e., the project staff should also be unaware of the vaccination status of the individual. The design should not be changed during the study, or if it is, the study according to the original design is to be considered as one finished study, and any continuation with a different design should be considered as a new study.

While these principles are all clear enough, it turns out that in practice there are many pitfalls. This is the subject of the following presentation. Partly this will be an eclectic listing of mistakes made, or barely escaped, in trials carried out in the past. The examples are largely taken from controlled trials of BCG, which are many and mostly well documented. Experiences from the recently concluded trial of BCG in the protection against tuberculosis, in the Chingleput District near Madras in South India, are very much taken into account.

[1] Received for publication on 22 November 1982; accepted for publication in revised form on 15 June 1983.

[2] J. Guld, Consultant, Tuberculosis Unit, WHO, Copenhagen, Denmark.

Record linkage

The basic statistical methods must be thought of well in advance. The use of a computer for all data processing is a must for even a moderately sized trial, such computers being available today in a vast range of capacities and at prices that are modest compared with a total cost of a trial. Punched card equipment is outdated, the data being inserted directly in hard or soft discs or tape from a visual display device. For a moderately large trial it is probably better to use a hard disc system, which is more expensive but also has a much larger capacity. Similarly, a line printer may be preferable to a (cheaper) matrix printer. At least one tape station may be useful, especially for long-term storage of data, for instance, between rounds of follow up.

The choice of a system of the correct size and configuration is thus to be studied well in advance with competent technical advice. The availability of dependable local service facilities is, of course, an important consideration. Duplication of the parts of the machine most likely to break down (e.g., the printer) may be worth considering. The alternative to very fast repair services may well turn out to be lengthy delays in the field work.

The record forms and shape of the information to be recorded in the field, and perhaps in the laboratory, should be elaborated well in advance and should be thoroughly tested in pilot studies. The size of each box in a form should depend on the amount of information likely to be entered (not on the printed headings, which can be in small print since the field staff will very soon know them by heart). The order of the boxes should be according to the logics of the field work (not to the statistical work, since the computer can easily rearrange the information). Similarly, the entries should be in a form con-

venient for the field staff. Thus the actual count of bacilli (in a direct smear) may be entered directly, since any desired reclassification can be executed by the computer.

The entries should be in a standardized form, easy to copy directly into the computer. The form may be numeric (e.g., age) but may just as well be alphabetic (e.g., M or F for sex). Lengthy, non-standardized entries (remarks) should be kept to a minimum, since they will have to be recoded by a statistical clerk or even by the statistician before they are entered in the computer. The standard codes for entries should also be worked out well in advance and be tested in pilot runs.

In general, a field record form should not be reused (e.g., for follow up) after the contents have been entered in the computer. Rather, the computer should print out new cards with the necessary information (individual number, name, sex, age and, perhaps, requests for additional examinations) for each follow up round. This will also contribute to the blindness of the study.

For the purpose of such computer-printed cards, but also for the purpose of identification of the individual, it is extremely desirable to record individual names in the computer. Unfortunately, computers available in the market nearly all have the Latin alphabet only and are limited to the 26 signs of English spelling. Thus, in a language that does not already have an authorized spelling with Latin characters, it may be necessary to construct one. To the extent possible, this should be a phonetic spelling, entirely without regard to English spelling (English spelling being exceptionally unphonetic) but, if possible, closely connected with the pronunciation and spelling of the local language. With only 26 characters at hand, special combinations of two characters may be used, such as "uu" for the vowel in "boot" and "u" for the vowel in "could." Or characters such as "/", "+", "−" may be introduced to represent particular sounds or modifications of sounds. Literate field workers with even a superficial knowledge of Latin characters will soon enough learn such a spelling system.

Identification of the individual

Each participant should be given an individual number to facilitate the bringing together of data from different sources for the same individual, especially as carried out by the computer. The individual number should be repeated on every separate record form.

In a trial based on registration by place of residence, the main identification may be by household or family: each household is given a number and the registration is done family-wise, so that the individual's identification can later be verified through the names of several family members (not only father's name, but also mother's, older brother's, etc.). The pattern of names in a society will, of course, have to be studied in advance; the system may be weaker in cases where some names are extremely common.

In the South Indian trial a fingerprint (left index finger) was obtained from each individual at the time of vaccination (or allocation to the control group). With a little practice and the use of a magnifying glass, it is usually easy for a clerk to see whether two fingerprints are from the same person. In this case no attempt was made to classify the fingerprints; they were only used for final confirmation of identification already made by name, household composition, etc.

If the registration is made in an institution (e.g., by school classes) a safe identification may be more difficult, although registration of the father's name should help in distinguishing children with the same name in the same class.

Change of residence may be a problem in a long-term study. If a whole family has moved it may not be so difficult, but if a young man has taken work in a different village, or a young girl got married and moved to the husband's residence, the clerical work involved in maintaining follow up may be considerable. A crossreference to the original individual number will be essential; the allocation of a new number in addition may be practical.

In the trial in South India, the listing of individuals alphabetically by village, and separately also by household, turned out to be of great help. The alphabetical list gave the household numbers for individuals with a particular name and the household list then gave details of each family, permitting final identification. The alphabetical sorting was done by computer of course.

Allocation

Mathematical aspects. The allocation may be either completely random or it may be some form of stratification, i.e., balanced for each of several subgroups. Allocation completely at random means that each person is allocated independently of the allocation of other persons. This can be done in principle by throwing a coin for each person: heads means vaccination, tails means control. In practice, this is a tricky operation. A better one would be to go by a list of random digits: for instance, an uneven number means vaccination, an even number (including zero) means control. The disadvantage of simple random allocation is that the numbers of vaccinated and controls will nearly always be slightly different. The importance of this disadvantage is, however, easily overrated, especially in the case of a large study population. The advantage is that a valid statistical analysis (analysis of variance) is quite without complications, whether the total population is considered, or subgroups of it. Should it turn out, after the event, that there is an interaction that might have justified a stratification (say, the protective effect of the vaccine depends on the age of the individual or on some other attribute), very little need have been lost since such a feature may be taken into account by the statistical technique known as analysis of covariance.

Stratification can be carried out in a number of ways. One rather naive way is to vaccinate every second person in the order they are registered. Another (often used in clinical trials in small study populations) is to define matched pairs, i.e., pairs that are alike in terms of severity of disease, age, sex, etc., allocating at random for each pair one person to be treated and one to serve as control. The advantage of stratification is obviously that the two groups, treated (vaccinated) and controls, will be more alike in composition. For example, if in a study in a school population every second child is vaccinated, the two groups will be more alike in terms of age distribution than would be the case using complete randomization: the children would usually appear one class at a time, i.e., one half of the seven-year-old children and one half of the twelve-year-olds, would systematically have been vaccinated. There would in other words be stratification on age. The disadvantage of any such stratifications is of a rather more subtle nature. The stratification will have to be taken into account in carrying out the analysis of variance, since otherwise the experimental error may be overestimated. A presentation of this problem, in non-mathematical terms, is offered by Sir Ronald Fisher ([2]). Such a complicated analysis of variance may be difficult, and the statistician is therefore often tempted to analyze the study as if it had been completely random. In conclusion, complete randomization is preferred unless there are very strong reasons for stratification. Such strong reasons will very rarely exist in the case of large-scale vaccination studies.

Allocation by "cluster" may be tempting, i.e., identical treatment (vaccination, or no vaccination) to all members of a household or even a village or a school class. However, this procedure is better avoided, not only for theoretical reasons (the experimental error will be larger and the statistical analysis more difficult) but also because the follow up may be less double-blind if the field staff know that all members of a cluster will have been uniformly treated.

Practical aspects. In a large vaccination trial it will not be possible to have a professional statistician to supervise the allocation for every member of the study group. Allocation will be made by nurses, paramedical workers, statistical clerks, etc., and such staff will have to take care of unexpected practical problems. Two examples from BCG trials of the past may serve as illustrations. When Aronson ([1]) started a trial in American Indians in 1935, the allocation had been registered in advance on the individual cards. Unavoidably, it happened that a child allocated for vaccination did not turn up. The field staff, on their own initiative in replacement of each such non-available child allocated for vaccination, vaccinated a child allocated to the control group; they did not, in return, withhold vaccination from a child allocated for vaccination if a child allocated to the control group failed to turn up. When in 1950, and later, Frimodt-Møller carried out a BCG trial in a South Indian population ([3]), the allocation was also made in advance by putting a cross on the back of every second individual card.

The next year the intake was continued by calling in individuals who had failed to turn up the first year and also by registering additional individuals. In the process, individuals failing to turn up the first year as controls were confused with individuals registered in the second year only. In hindsight, this confusion could have been avoided by putting zeros on the back of individual cards allocated for control.

Recording of the vaccination of the individual (or allocation to the control group) should obviously be done with a minimum of mistakes. Normally it will be justified to have an assistant to the vaccinator, who will dictate or check the allocation of the individual and record the fact of the vaccination having been performed or withheld.

Another problem is the treatment of children with an absolute indication for vaccination (in the case of BCG, this might be known contacts of open cases of tuberculosis), or a relative or absolute contraindication (fever, known immunodeficiency). In these cases, it is imperative that the responsible field worker decides without reference to the allocation to vaccinate or not to vaccinate, as the case may be, and makes a corresponding record on the card so that all such cases can be excluded in the course of analysis. Otherwise, the hypothetical protective effect of the vaccination might be confounded with the increased risk for the contacts, and the protective effect be underestimated.

Blind and double-blind procedures

The use of a placebo procedure has become increasingly unpopular in recent years for what is called "ethical" reasons. This being a political matter, there is nothing for the investigator to do but to abide by the rules and decisions in force locally.

The placebo (which may be an injection of isotonic saline or the intake of an innocuous pill) serves two purposes. One is to contribute to the blindness of the study, in that the individual himself will be less aware or unaware of his treatment status. The other is explicitly to define the study population: while the person allocated to vaccination but not turning up (or refusing) is easily excluded from the study population, the corresponding person allocated to the control group but failing to turn up is less easily defined. With the use of placebo such persons are easily defined as those that have not received the placebo. Without the placebo, there must be strict rules for recording non-attendance—not a simple thing in case the team makes several attempts to see such a person.

If the use of a placebo is not permitted, it may be possible to obtain a fair degree of blindness by offering an additional procedure to all, for instance, a triple vaccination. The individual is much less likely to notice and especially remember whether he has received one or two pricks, than he is to remember whether he has received one or none. There remains, of course, the rare case of the person who accepts the first injection but refuses, one minute later, the second injection.

It may be tempting to offer an injection of assumed benefit (e.g., a triple vaccination) to the control group only, so that all participants receive one and only one injection. In principle, however, this may be objectionable because it means that the vaccinated group and the control group are treated differently both in a relevant and in a (hopefully) irrelevant way.

REFERENCES

1. Aronson, J. D., Aronson, C. F. and Taylor H. C. A twenty-year appraisal of BCG vaccination in the control of tuberculosis. Arch. Intern. Med. **101** (1958) 881–893.
2. Fisher, R. Systematic squares. In: *The Design of Experiments.* 6th ed. London: Oliver and Boyd, 1951, section 34.
3. Frimodt-Møller, J., Thomas, J. and Parthasarathy, R. Observations on the protective effect of BCG vaccination in a South Indian rural population. Bull. WHO **30** (1964) 545–574.
4. Trial of BCG vaccines in South India for tuberculosis prevention. Tuberculosis Prevention Trial, Madras. Indian J. Med. Res. **72** Suppl. (1980) 1–74.