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SPECIAL PAPER

Report on the First Meeting of the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) Consortium held at Armauer Hansen Research Institute, ALERT, Addis Ababa, Ethiopia on 24–27 October 2004

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BEHALF OF THE IDEAL CONSORTIUM

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Introduction

The IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) consortium was established following a WHO/TDR sponsored workshop held in Amsterdam in October 2003, in which strategies for the development of new diagnostic and epidemiological assays, based on these technical developments, were defined. An Interim Steering Committee was appointed with the objective to set up a network of relevant research and field sites, write a coordinated research proposal and, where possible, coordinate activities between partners. A call for partners was published at the end of 2003 via the Leprosy Mailing List. At the moment the network consists of nearly 30 partners from all continents.

The Heiser Program for Research on Tuberculosis and Leprosy of the New York Community Trust funded the organization of a workshop with all partners of IDEAL. The meeting comprised state of the art lectures and workshops, which are reported here.

Background

Early detection and treatment is the key to successful control of leprosy. This approach not only interrupts transmission of the bacterium, but also prevents the development of nerve damage, which leads to disability and handicap, and often also to social isolation and destitution.

The diagnosis of the disease is still largely based on clinical symptoms and only occasionally verified by examining skin slit smears for the presence of acid-fast bacilli by light microscopy. However, increasingly fewer centres have trained microscopists and, furthermore, microscopy lacks sensitivity and specificity, being unable to distinguish

between the various mycobacteria that are pathogenic for humans. The availability of a specific, diagnostic method has long been desired. Ideally, such a method would enable the detection of infection and the presence of multiplying *Mycobacterium leprae* at early stages, before clinical signs leading to leprosy become apparent or transmission occurs.

Recently, methods for strain differentiation have become available based on the detection of single nucleotide polymorphisms or variable numbers of tandem repeats at certain loci within the genome of *M. leprae*, allowing for the first time molecular epidemiological studies into the transmission of disease. A robust typing system would clearly benefit leprosy control efforts through better understanding of how the organism is transmitted and should ultimately lead to the development of methods that effectively reduce the number of new cases of leprosy and/or interrupt transmission. Secondly, definition of the genome of the TN strain of *M. leprae*, combined with the application of comparative genomes, allowed the recognition of some 31 *M. leprae* specific proteins. The application of these, and particularly their peptide epitopes, in assays based on cell-mediated immune responses has allowed the detection of *M. leprae* infection, selective from infection with *M. tuberculosis*.

Strategies for diagnostic assays, state of the art

Hazel Dockrell (London School of Hygiene and Tropical Medicine, London, UK) outlined the approaches that have been used to identify *M. leprae*-specific T cell antigens, which would provide specificity comparable to phenolic glycolipid I (PGL-I), which is recognized by antibodies. These include:

- The fractionation of *M. leprae* to provide cell wall, cell membrane, and cytosolic antigenic preparations (P.J. Brennan); derivations of some of these are currently being tested as skin test reagents in Nepal (M. MacDonald).
- The earlier WHO Synthetic Peptide Task Force Initiative.
- Current studies on new recombinant antigens and peptides.

Dr Dockrell concentrated on the latter two approaches. The WHO Synthetic Peptide Task Force was a multi-centre study involving one non-endemic (UK) and four leprosy endemic countries (Brazil, Ethiopia, Nepal and Pakistan) in which 81 peptides from *M. leprae* proteins were tested for T cell recognition. Although some of the peptides tested gave promising results, most of these selected for the study were subsequently found to be very homologous to equivalent sequences in *M. tuberculosis* when additional genomic information became available. However, the co-ordinated testing approach revealed interesting differences in peptide recognition in different ethnic groups.

Accordingly, new T-cell reagents should first be screened for homologies to *M. tuberculosis* and other mycobacteria such as *M. bovis* BCG and *M. avium*. Such antigens should induce a T cell response in paucibacillary (PB) leprosy patients, and a high proportion of leprosy contacts, but not in TB patients, or non-endemic controls; there should also be higher proportions of responders in endemic settings with high endemicity than in those in countries with lower endemicity.

Ideally, new T cell diagnostic assays would provide complementary coverage to a serological assay. Once identified, *M. leprae*-specific T cell antigens could be used in studies

to distinguish subjects with protective immunity, for example those who had self-cured, from those developing the disease; the latter should be given prophylaxis.

Stewart Cole (Institut Pasteur, Paris, France) reported on recent work with R. Araoz to identify *M. leprae*-specific T cell antigens. Despite the genome downsizing in *M. leprae* relative to *M. tuberculosis* (3.3 Mb compared to 4.4 Mb) there are 165 genes that appear by comparative bioinformatics to be *M. leprae*-specific.

Twenty antigens were produced as purified His-tagged recombinant proteins: 13 from genes thought to be unique to *M. leprae*, five from common mycobacterial genes and two from secreted/exported antigens. The recombinant antigens were tested for their ability to induce IFN- γ production in whole blood cultures from 12 leprosy patients [8 PB and 4 multibacillary (MB)], 12 TB patients, and 12 leprosy contacts, at CNAM (Bamako, Mali, with Dr S. Sow). Mali had a reported leprosy prevalence of 1.47/10,000 in 2000, with 1786 new cases. The recombinant antigens induced strong IFN- γ responses in the leprosy patients, moderate responses in TB patients and leprosy contacts in Mali, and weak responses in Parisian blood donors, used as non-endemic controls. Some of the new antigens were also recognised by circulating antibodies. Some of these antigens may therefore have potential as T cell and B cell diagnostic reagents.

Tom Ottenhoff (Leiden University Medical Center, Leiden, the Netherlands) reported work performed by Annemiek Geluk together with Elizabeth Sampaio (FIOCRUZ, Rio de Janeiro, Brazil). The *M. tuberculosis* ESAT-6 and CFP-10 proteins are useful diagnostic antigens in tuberculosis, as their genes are deleted from the genome of *M. bovis* BCG. However, the *M. leprae* ESAT-6 and CFP-10 proteins proved to be cross-reactive and recognized by TB as well as leprosy patients.

Genes from 17 *M. leprae*-unique open reading frames (ORFs), encoding proteins of unknown function were selected, with a protein size of >70 amino acids, <30% homology with *M. tuberculosis*, and that contained binding motifs for >75% HLA class II alleles. The recombinant antigens were tested for their ability to induce IFN- γ secretion in cultures of peripheral blood mononuclear cells (PBMC) from 127 Brazilian subjects: 28 PB and 19 MB leprosy, 34 *M. leprae*-responsive leprosy contacts and endemic controls, 30 non-*M. leprae*-reactive endemic controls and 16 TB patients, (five of whom responded to *M. leprae*). Five of the new antigens showed remarkable specificity and were only recognized by leprosy patients (some of whom were in reaction), *M. leprae*-reactive contacts and *M. leprae*-reactive TB patients. In all, 94% of anti-PGL-I antibody-negative PB leprosy patients, and 61% of the anti-PGL-I antibody-negative *M. leprae*-reactive contacts/controls induced a T cell response (IFN- γ) to at least one of these five *M. leprae* unique candidate antigens.

These new antigens therefore have great potential as diagnostic reagents, and could be used in longitudinal studies of leprosy contacts to identify biomarker profiles predicting protection or progression to clinical disease; they may also have value in the prediction of leprosy reactions.

John Spencer (Colorado State University, Fort Collins, USA) reported on progress towards the development of *M. leprae*-specific skin test antigens for leprosy, currently in phase II clinical trials in Nepal (see presentation by Dr Murdo Macdonald). The fractionation of *M. leprae* to provide cell wall and cytosolic antigenic preparations shows promise but as these fractions still contain many cross reactive mycobacterial antigens, further work is focusing on the identification of *M. leprae*-specific antigens.

From ORFs predicted to be *M. leprae*-specific, 28 genes were selected, and four tested as recombinant antigens; 58 synthetic peptides encoding predicted T cell epitopes were also synthesized, derived from 13 unique and 13 other *M. leprae* genes. The recombinant antigens and peptides were tested for their ability to induce IFN- γ secretion in PBMC cultures from leprosy patients, contacts and controls in Brazil by Cristina Pessolani.

Cristina Pessolani (FIOCRUZ, Rio de Janeiro, Brazil) described the results of these experiments. Although the genes of these antigens were not strongly expressed by *M. leprae* in comparison to those for five other antigens, such as GroES, one of the antigens was recognized only by leprosy patients and contacts and not by TB patients or non-endemic controls, while the others gave greater recognition in the endemic controls and TB patients. Many of the peptides (35/58, 60%) showed excellent specificity, only being recognized by leprosy patients and their contacts. There were indications that some peptides were recognized more strongly by PB patients than by leprosy contacts, while others showed the opposite pattern of recognition. Flow cytometric analysis showed that some of the antigens and peptide mixtures induced the activation of CD4 T cells that expressed the early activation marker CD69. Thus, some of these synthetic peptides may be useful for the diagnosis of leprosy and prediction of disease.

Murdo Macdonald (Anandaban Leprosy Hospital, Kathmandu, Nepal) reported on the challenges involved with the field-testing of the new diagnostic skin test reagents in Nepal, where the new case detection rate is 2.84/10,000. These include cultural sensitivities towards pregnancy testing (pregnancy was an exclusion criterion), and the definition of contacts in an endemic region. Before performing such studies, the necessary permissions must be obtained from a number of regulatory bodies in the USA and Nepal. It is also difficult to obtain true informed consent in a setting where illiteracy is high.

In the current skin test studies, the numbers of healthy non-exposed Nepalis tested were increased from 10 in part A to 90 in part B; no reaction was detected to diluent and the new antigens only induced minimal reactions at the higher doses. In part C, 147 Nepali leprosy patients, 140 leprosy contacts and 48 TB patients will be tested with the two leprosy skin test reagents, MLSA and MLCwA, and PPD, injected intradermally.

Skin tests, serological tests and *in vitro* whole blood tests for cytokine production all have their advantages and disadvantages, but could be designed to exploit new, *M. leprae*-specific, antigens to provide new diagnostic tests for leprosy.

Paul Sanderson (American Leprosy Missions, ILEP) outlined the potential uses of new *M. leprae*-specific antigens in field studies. For clinical diagnosis, it is important to identify individuals with active disease, with subclinical infection, but not those who have been cured with drugs or who have self-healed. For epidemiological purposes, it would be useful to know what proportion of the population has been exposed to, or infected with, *M. leprae*. Antibody tests may miss many PB cases, while T cell based tests may miss MB cases. Assays for antigen detection may have similar drawbacks as serological assays, but might be better at distinguishing active from previous infection. It is difficult to determine the specificity of a new test, as this requires large numbers of subjects known not to have leprosy, and this would require long term follow up of such subjects. For a rare disease like leprosy, even with a specificity of 99.9% the number of individuals identified as positive in a new test would be far greater than the numbers of new cases normally identified clinically, and such overdiagnosis

would not be acceptable – it may be better to pre-screen subjects so that only those suspected of having disease are tested.

Willem Hanekom (University of Miami, Miami, USA) described a large-scale study based in Worcester and Cape Town, South Africa (with Greg Hussey, University of Cape Town), designed to identify immune correlates of protection against TB induced through newborn BCG vaccination. These might include specific CD4, CD8 and regulatory T cell responses and additional components identified by approaches such as microarrays, and antibody responses.

Heparinized whole blood samples are stimulated with BCG and antibodies to co-stimulatory molecules for 7 h, brefeldin A added for a further 5 h followed by harvesting, lysis of the red cells, fixation and freezing in liquid nitrogen. After thawing, permeabilized cells are stained for surface phenotypic markers and for intracellular cytokines. Such assays are very dependent on the time taken to get the blood into culture, and so blood samples are added in the field to tubes containing antigen and transported to the main laboratory in portable incubators.

There was a good correlation between soluble IFN- γ and the proportion of CD4 T cells with intracellular IFN- γ . BCG also induces CD8 T cell activation, with a slightly weaker association between CD4 and CD8 activation in individual subjects. Anti-CD107, or antibodies to granzyme A or perforin can also be used to quantitate cytotoxic CD8 T cells. To quantitate regulatory T cells, real-time RT-PCR can be used to measure mRNA for FoxP3; perhaps surprisingly, FoxP3 expression was correlated with IFN- γ production, and with TGF- β rather than IL-10 expression. Diluted whole blood assays can also be used to assess the functional capacity of cells, for example by measuring BrDU incorporation on day 6 to assess the proliferation of particular T cell subsets. There was, however, a striking lack of correlation between 12 h CD4 T cell production of IFN- γ and the proliferation of CD4 T cells at 6 days, highlighting the need to compare shorter and longer term assays of T cell function.

Overall, these results indicate the many parameters of T cell activation that can now be assessed using flow cytometric analysis.

Hazel Dockrell (London School of Hygiene and Tropical Medicine, London, UK) reviewed the literature on assays that are being used in the TB field with *M. tuberculosis*-specific antigens, to assess infection in TB patients and their contacts. These include skin tests, measuring cytokine production, lymphocyte proliferation, and quantitating antigen-specific T cells using tetramers; other parameters could be assessed using real time RT-PCR. Synthetic peptides have been used as a skin test reagent in HIV. PBMC assays have been shown to be suitable for measuring IFN- γ production induced by peptides, and responses to peptide pools correlate well with those to recombinant antigens. Pools of peptides from the *M. tuberculosis*-specific ESAT-6 and CFP-10 proteins have shown good specificity in ELISPOT assays with TB patients and contacts. Possible drawbacks of ELISPOT assays include the rather spiky time kinetics of such *ex vivo* responses, for example following vaccination (although this might make the assay a better indicator of active infection), and the need to get the blood into culture rapidly, as with the intracellular cytokine technique.

Diluted whole blood assays stimulated for 6 days with mycobacterial antigens have been used in studies of responses to BCG vaccination in Malawi and the UK; such responses correlate with the Mantoux skin test response, which may indicate that such IFN- γ production is not a correlate of protection. The commercially available QuantiFeron assay is a whole blood assay in which undiluted whole blood is stimulated with mycobacterial antigens overnight; in TB, this assay works well with a pool of specific *M. tuberculosis* peptides.

Further cytokines can be detected using the newer multiplex cytokine assays, although for field use a simpler method of IFN- γ detection would be preferable. Some cytokines cannot be detected easily in culture supernatants, and some, like IL-4, may indicate progression towards clinical disease in TB contacts. Real time RT-PCR can also be useful for detecting such cytokines, and for discriminating between IL-4 and its antagonist, IL-4d2. Lymphocyte proliferation can now be well assessed using flow cytometry, with CFSE or BrDU incorporation. Flow cytometry can also be used to quantitate individual peptide-specific cells, although these techniques work better for CD8 than for CD4 T cells, and the variability of HLA types in different ethnic populations may be a problem.

Paul Klatser (KIT Biomedical Research, Amsterdam, the Netherlands) presented an overview of serology. Several studies have now clearly established that anti-PGL-I antibody positive household contacts of leprosy patients have a significantly higher risk of developing leprosy, notably MB leprosy, compared to seronegative contacts.

Although serology is not a universal marker for leprosy, it does discover patients with higher bacterial loads that are missed by slit skin smear examination. Seropositive PB patients have an increased risk for treatment failure. Many studies have illustrated that seropositivity is a better reflection of the total bacterial load than the bacterial index of the skin.

Close contact is more important in transmission than often believed. The risk of developing leprosy is greatest among close contacts of leprosy patients, like household contacts, but also neighbours and social contacts, and in particular among close contacts of MB patients. Screening contacts of leprosy patients in order to find and follow-up or treat those at increased risk of developing leprosy should ultimately prevent transmission and opens the way for a rational programme for eradication. Serology is a useful tool for this purpose. Recently, a simple lateral flow test for the detection of anti-PGL-I antibodies has been described, which can replace ELISA and extends the application of serology to local leprosy control programmes.

Diagnostic assays workshop

The discussions in this group focussed on immunological diagnostic assays, both antibody based as well as T cell based assays. It was generally recognized that both types of assays may be needed in the field to diagnose leprosy patients across the disease spectrum. The group first spent some time on the desired qualities of a diagnostic test and the following attributes were identified as key: specific, affordable, simple, safe, and field friendly.

Currently the following diagnostic tests for leprosy are being used or are at various stages of development: biopsies, skin smears for acid-fast bacilli (AFB), *M. leprae* PCR in the context of various body fluids, antigen detection (immunological), and anti-PGL-I antibody detection (immunological). Any of these tests would be used in combination with clinical examination.

ANTIBODY BASED ASSAYS

The only antibody-based assay available at the moment is the PGL-I antibody assay, which has been extensively evaluated in the field. The results clearly indicate that seropositivity is a major risk factor in development of disease with up to 40% of seropositive contacts going on to develop disease. This assay is also very effective in differentiating MB and PB disease in

leprosy. However, a large proportion of tuberculoid patients are seronegative in the PGL-I test and an additional second test that would provide higher levels of specificity/sensitivity is desired.

A strong recommendation was therefore made for screening all new recombinant antigens for identifying new targets that could be used in antibody based assays.

The following activity plans were put forward.

1. *Screening the new available recombinant proteins*

- Extend existing work on the applications of PGL-I.
- Screening for new potential antigens among the new available unique proteins/peptides.

2. *Standardization*

- Standard definitions of contacts (such as contact history) and clinical information on patients including reactional status.
- Build up a bank of serum samples coming from well defined individuals.
- Standardize protocols and procedures.
- Test development.
- Test evaluation/validation.

3. *Development of field friendly assays*

- Compare the two available lateral flow tests, those produced by KIT Biomedical Research in Amsterdam and by Dr Sang-Nae Cho at Yonsei University in Seoul.
- Development of lateral flow test for recombinant antigens.
- Make PGL-I tests more sensitive.

4. *Evaluation/validation*

- Test validation with defined sera.
- Test evaluation in field.
- Link to other fieldwork or intervention studies.

T CELL BASED ASSAYS

There are already several *M. leprae* unique antigens identified. It was generally accepted that testing of these antigens should be an immediate priority.

The following activity plans were put forward.

1: *Identification of T cell target antigens.*

There are more than 41 recombinant proteins already being tested in different sites. An additional 35 peptides are available for screening. The group therefore felt that these potential target antigens should be screened in different populations. Potential new targets that have been identified are 39 'unique' ORFs. The group also felt screening overlapping peptides may allow better identification of T cell epitopes. However it was also pointed out that there are now more sophisticated programs available for predicting T cell epitopes and both approaches should be kept in mind since it may not be possible to screen a large number of overlapping peptides for budgetary reasons.

2: *Screening of currently available antigens in different populations*

There was consensus within the group that IFN- γ is still the best biomarker available for predicting infection. There are several different assays being used for detection of IFN- γ in stimulated cells. These included PBMC stimulation, ELISPOT test, intracellular cytokine

staining and QuantiFeron-type whole blood assay. There was considerable discussion on the optimal screening test. There was a consensus among the group that stimulation of PBMC for 5 days and detecting IFN- γ in the supernatants is the best available assay for detecting specific memory T cell activation at the moment. It was also felt that testing in different sites would require considerable coordination in terms of standardization of protocols and procedures as well as handling of database. Currently the following endemic sites are actively participating in screening of recombinant antigens and unique peptides: Brazil, Pakistan and Nepal. Additional sites need to be identified. One of the major needs in this activity will be standardization of the IFN- γ protocol. One strategy that was discussed was that there should be a first line screening in a single laboratory to narrow the focus to a few specific peptides/antigens and then extend it to second line screening and explore their use in other communities/populations.

3: Definition of additional biomarkers

After the final selection of the most promising specific targets in T cell assays, it was felt that these antigens could be tested for a range of other markers which are associated with protection and/or pathology. The biomarkers that were felt to be of interest were IL-10, IL-4, TGF- β and TNF- α . Understanding the development of disease was felt to be an important issue and animal models such as the monkey and the armadillo model could be further explored. These studies should also allow development and exploration of promising new technologies such as BD multiplex bead assays, real time RT-PCR, arrays and multiparametric flow cytometry (activation markers, CD4 and CD8, BrDU) to address fundamental questions in the biology of disease.

4: Development of a field-friendly test

It was felt that this could be best done in collaboration with commercial partners with expertise such as with the QuantiFeron manufacturer who has recently marketed a field friendly assay for detecting latent infection in tuberculosis.

5: Validation/evaluation in the field

The following issues were identified as important: supporting roles and other issues, adherence to procedures/protocols, statistical issues, data management issues, other issues relating to the move from research to manufacture, and social science input.

6: Social support package

Facilitation, community liaison, and counselling.

Molecular epidemiology, state of the art

Stewart Cole (Institut Pasteur, Paris, France) began the molecular epidemiology session with a presentation reviewing our current understanding of the composition and organization of the *M. leprae* genome. He surmised that gene decay through selective pressures has produced a bacterium with 'just enough' genes to survive as a successful pathogen. He presented results from comparative genomic analysis with other mycobacterial species identifying approximately 130 *M. leprae*-specific genes with potential for development in the area of diagnostics.

Dr Cole presented recent evidence from his laboratory suggesting that *M. leprae* is highly clonal and a recently diverged human pathogen. These conclusions were based on analyses of a significant number of *M. leprae* isolates from around the world using:

- DNA macroarray analysis in which no insertions or deletions were observed.
- Quantitative PCR based on repetitive DNA sequences (REPLEP, LEPREP, LEPRPT, and RLEP) showing no copy number variation among isolates tested.
- Sequence analysis of a single mycobacterial interspersed repetitive unit (MIRU) showing no diversity.

Single nucleotide polymorphism (SNP) analysis further supported the highly clonal nature of the global isolates tested and revealed four major groups of *M. leprae*. A model for global spread of *M. leprae* was presented based on SNP markers suggesting possible origins and historical/temporal relationships pertinent to the dissemination of leprosy worldwide.

Finally, results from studies using a battery of variable number tandem repeats (VNTR) suggested that, because of the observed extreme degree of variability, certain of these repetitive elements may best be used for monitoring transmission with close proximity relationships.

Shinzo Izumi (Airlangga University, Surabaya, Indonesia) summarized epidemiological studies performed in Indonesia over the last few years. Leprosy in Indonesia, as in many countries, displays an uneven distribution with pockets of disease reaching prevalence rates as high as 4-25/10,000. Areas under study had prevalence rates greater than 2/10,000, PGL-I serological positivity in the range of 40% and PCR positivity rates for *M. leprae* from nasal secretions around 27%.

Studies in villages were undertaken to learn more about the distribution of *M. leprae* isolates using the VNTR strain marker TTC. Stability testing of the TTC repeat was performed by serial passage of 11 strains of *M. leprae* in nude mice and showed that TTC repeats were stable over time, supporting their applicability to epidemiological studies. *M. leprae* from both nasal secretions and skin slit smears were used in testing for TTC repeats. Results showed that the genotypes of *M. leprae* recovered from the nasal cavity of various family members were discordant, suggesting that exposure in these villages may result from multiple sources. TTC testing of *M. leprae* isolated from new cases in five households harbouring a prior case of leprosy showed that only three household contact cases were infected with *M. leprae* carrying the same number of TTC repeats as the index case. *M. leprae* TTC repeats from the other two household contact cases were not concordant with the respective index cases, suggesting that exposure of household contacts is not solely to *M. leprae* from the respective index case and that other sources of transmission should be considered.

Local water testing was performed to investigate a potential environmental site for contamination with *M. leprae*. Identification of *M. leprae* DNA in water samples where individuals bathe was confirmed suggesting the possibility that environmental sites may be involved in transmission of leprosy in some Indonesian villages.

Varalakshmi Vissa (Colorado State University, Fort Collins, USA) summarized the rationale for using multiple locus VNTR analysis (MLVA) in studying the epidemiology of infectious diseases and gave examples of their utility in understanding various aspects of diseases caused by *M. tuberculosis*, *M. bovis*, *S. typhimurium*, *F. tularensis*, *E. coli*, *B. pertussis* and *Y. pestis*. She described the technology that has been developed for analyzing MLVA and showed an analysis of VNTR typing using a panel of nine isolates of *M. leprae* and 25 separate VNTR loci. The isolates were from India, Brazil, USA and Thailand and MLVA showed discriminatory power at most VNTR loci indicating a rich array of possible markers for use in epidemiological studies.

Using MLVA, she also studied the genetic relationships of unknown *M. leprae* isolates archived from *M. leprae*-infected armadillo tissues. Some isolates did not have a clear pedigree when compared to known *M. leprae* isolates. However, in one case MLVA comparisons made possible reasonable linkages between one isolate from archived tissues to the TN strain of *M. leprae*. This application suggests that various VNTR loci may be useful for strain linkages and, therefore, useful for transmission studies.

Another important point that emerged from her data was that the larger, less frequently occurring minisatellite VNTRs showed fewer disparate alleles throughout the *M. leprae* isolates studied. These observations make clear that selection of a minimal set of VNTR alleles useful for epidemiological purposes may require different combinations of VNTR loci to address specific questions regarding transmission or other questions about the disease ecology of leprosy. Both highly variable VNTR loci as well as less variable loci may be required and further study of these and other regions of the *M. leprae* genome are required.

She also concluded from her studies in China and Columbia that DNA from skin slit smears and nasal secretions is amenable for VNTR typing but yields and PCR efficiency are limiting factors. Finally, it appears from her work that multiplexing reactions may be a possibility that would reduce the need for large amounts of patient material needed for study.

Sang-Nae Cho (Yonsei University, Seoul, South Korea) presented results from molecular typing studies conducted in Cebu, The Philippines. Three markers (GTA, GCACCT and TTC) were used to type isolates of *M. leprae* from epidemiologically linked MB patients. The frequency of alleles for each VNTR was established in the communities and showed that TTC had the greatest allelic variability and GCACCT the least. Of 59 patients studied 41 distinct types of *M. leprae* were identified. Of 17 village clusters examined only eight (47%) had similar molecular types of *M. leprae*. Of two family clusters examined none were completely matched. Finally, in three cities ten major types of *M. leprae* were identified originating from 56 patients.

Molecular epidemiology workshop

The working group discussed the state of the art in terms of available tools to investigate the molecular epidemiology of leprosy. Tom Gillis summarized the presentations on the first day of the workshop by the different groups from the point of view of field application. The group agreed on the agenda and procedures for further discussion.

The rationale for genotyping *M. leprae* is to understand transmission to better focus intervention strategies to halt transmission. Clarifying historical, geographical and phenotypic relationships of *M. leprae* may inform our understanding of the overall disease process in leprosy.

Genotyping *M. leprae* has the potential to become an effective tool for clinical and epidemiological applications. A robust typing system would clearly benefit leprosy control efforts through better understanding of how the organism is transmitted and ultimately lead to the development of methods that effectively interrupt transmission and reduce the number of new cases of leprosy.

CURRENT CONSTRAINTS

On a closer look at the current state of the markers in the published literature and data presented for the first time at the workshop, it was appreciated that the available tools could

already answer certain questions related to the evolution and migration of the pathogen globally (SNPs) and, on the other hand, demonstrate extensive diversity among strains in a relatively small group of patients (VNTRs). However, in order to effectively track transmission in close range, it is necessary to acquire tools with the appropriate resolution, discriminatory power and stability that the research question would demand. Work is ongoing in many laboratories to further characterize potential markers in multiple loci of the *M. leprae* genome. There is need to continue to screen for VNTRs particularly in intragenic areas and to discover new target sequences suitable for clinical and epidemiological application.

Lack of uniform protocols, nomenclature and standard study materials was an important concern of the group. It was highlighted that a uniform panel of *M. leprae* template with known numbers of animal passages, and same standard operating procedures was required. Data gathered from these panels could be shared on a web page for group use. Protocols (number of 'runs', extraction protocols) for work on various types of human samples (biopsy, nasal secretion, skin lymph and others) could constitute such postings. It is essential to establish a minimum set of markers for a robust typing scheme. Diversity panels could be distributed from a centre (such as the National Hansen's Disease Programme, USA) as blind specimens for purposes of standardization of test systems.

FIELD TESTING

In the area of field-testing, the group discussed the various options available in terms of possible field sites and listed possible selection criteria. These included the number of patients in a period of time, proportion of MB cases, availability of laboratory facilities, documentation, sample storage or archiving, communication, trained/experienced manpower, etc. The choice of study sites could be determined by the relative need for geographical diversity (global, regional, local), endemicity level, socio-demographic and cultural variables and other risk factors relevant to the study design in question. The significance of identifying relapse or re-infection was debated. It was argued that, as long as the organisms were still susceptible to the chemotherapy, both conditions posed no real threat to leprosy control. Studying the transmission among family contacts was identified as one important application of the molecular tools.

SAMPLES

The group discussed various alternatives for sample collection and sharing. It was appreciated that many centres have archival material and ongoing research work that could serve as source of specimens for the planned study. The need to have appropriate ethical clearances for collection of patient specimens was stressed. Most of the molecular work could be done at the sites where samples are obtained. There was however a need for links with state of the art laboratories for data analysis and advanced investigations. Capacity building in the field sites will be an important activity among the partners.

It was suggested that archival material could serve in the characterization and validation of the tools and provide a pattern for epidemiological 'reading' of the diversity of *M. leprae* at a field site. Prospective studies on patient samples from the same area could then be compared ('plugged into') to see how these fit into the identified pattern.

The group agreed that the best specimens for molecular epidemiology would be 4 mm punch biopsy specimens placed in 70% ethanol (avoiding formalin), sectioned to no more than

2–3 parts. However, skin smears rinsed into DNA preservative medium were also reported to be satisfactory. Whole blood was also mentioned as a possible source of *M. leprae* DNA.

Other issues that the group looked into included animal passages for stability testing of target sequences, studies into drug resistance loci and human susceptibility genes. Drug resistance was not considered a problem at the moment. Not all mutations translate into *in vivo* resistance as well. The use of patient samples for human genome studies could raise ethical concerns.

OUTLINE FOR RESEARCH

The group formulated an outline for research. The suggested schedule was to focus on marker development in the first year in the state of the art laboratories already active in the area, targeting more sequences in the *M. leprae* genome, in addition to exhaustively characterizing those already in the pipeline. Parallel and following the development of the tools, work should proceed on standardization of definitions, standardization of laboratory methods and validation of the tools on a defined (preferably) panel of *M. leprae* isolates from various original sources, including from animal passages and fresh human samples. Multiple specific research questions will be formulated and addressed in the course of these efforts.

The findings from these investigations will then lead, in the 3rd year of the project, to the development of hypotheses on transmission of *M. leprae* for field-testing using the newly validated tools, the outcome of which would be expected to impact leprosy control as a result. In preparation for the field study and parallel to it, the group stressed the need for capacity building and closer interaction of the partners through repeated meetings, workshops, as well as a dedicated web page that could be hosted by International Leprosy Association or other sites, and would serve as a repository of information for group use.

Steering Committee

The Steering Committee for IDEAL was elected and now consists of the following persons: Professor Patrick Brennan (chair), Professor Hazel Dockrell (vice-chair), Dr Linda Oskam (secretariat), Dr Abraham Aseffa, Professor Rabia Hussain, Dr Abraham Joseph, Dr Jan Hendrik Richardus, and Dr Euzenir Sarno.

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Annex: list of participants

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2	Patrick Brennan	Colorado State University, Fort Collins, USA
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6	Sang-Nae Cho	Yonsei University, Seoul, South Korea
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8	Hazel Dockrell	London School of Hygiene and Tropical Medicine, London, UK
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