Report on the Workshop on New Diagnostic Tools for Leprosy

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The purpose of the Workshop was to assemble the expertise to define where we are today and where we should be five years from now in terms of developing new tools for the diagnosis of leprosy over and beyond clinical examination, skin smears, and histopathology. The greatest priority was for early diagnosis and surveillance/epidemiological monitoring of leprosy. Other priorities were for tests that predict reactions and drug resistance. The discussions centered on serology, assaying for drug resistance, PCR, *in vitro* assays for γ-IFN, skin testing, and new antigens for diagnosis in general.

The Strengths and Limitations of Serology. Present-day serological diagnosis of leprosy relies solely on detecting IgM antibodies to PGL-I using a semi-synthetic neoglycoconjugate surrogate of PGL-I. Such serology in the past relied on ELISA. In recent years, there have been major developments in the creation of kits, such as the ML dipstick and a 10 min Leprosy Lateral Flow Test Kit. The concordance with conventional ELISA is excellent. In general, paucibacillary (PB) leprosy patients show a positivity rate of less than 20% compared to >90% for multibacillary (MB) leprosy. These kits are useful for identifying household contacts at risk who may represent subclinical leprosy. Ease of use of these devices recommends them for aspects of control programs. Prospects for antigens with serological sensitivity and specificity greater than PGL-I are dim. PGL-I in its various formats is probably the best serological tool for leprosy that can be developed.

Tools for Detecting Drug Resistance. Rifampin, dapsone, and clofazimine currently constitute the components of the WHO-recommended multidrug therapy (MDT) for both MB and PB leprosy. In addition, ofloxacin and minocycline can be used as second line drugs for treatment of drug-resistant leprosy or, more recently, in conjunction with rifampin (ROM) as a single dose therapy for the treatment of single lesion, PB leprosy. The rates of antileprosy drug resistance are unknown in many countries of the world due to the inability to rapidly determine drug susceptibility in *M. leprae*. The current mouse footpad (MFP) drug susceptibility assay requires large numbers of mice (20–40) and 6–12 months to obtain results. Therefore, there are only a few laboratories in the world that presently perform the MFP drug susceptibility testing. One of these is the Schieffelin Leprosy Research and Training Center. From 1987 to 1997, 265 leprosy biopsy samples were evaluated for resistant strains of *M. leprae* in the mouse footpad. Of these, 49 (18.5%) were resistant to varying concentrations of dapsone, rifampicin, or clofazamine, and, of this number, 23 (47%) of these were from a control area. With 369 skin smear-positive MB patients as the risk group (denominator), 23 (6.23%) were resistant to one or more drugs, 18 (4.88%) had dapsone resistance, 5 (1.36%) were resistant to rifampicin, and 9 (2.44%) had resistance to low concentrations of clofazamine (0.0001%). Out of the 23 biopsies from the control area with drug resistance, primary dapsone resistance was seen in 7 (30%) biopsies and secondary dapsone resistance in 11 (48%). Primary rifampicin resistance was seen in 4 (17.4%), secondary rifampicin resistance in 1 (4.35%) patient and primary clofazamine resistance in 7 (30%) biopsies. Three (13%) of the strains showed secondary clofazamine resistance. One biopsy had resistant strains to all three drugs. In an area where properly supervised effective multi-drug therapy (MDT) was regularly adminintered, over the years, the emergence of drug resistance is neglibible. However, this may not be the case if the content, duration, and regularity of the drug regimen are not satisfactory. Knowing the

possible shortcomings of mass administration of MDT, this study suggested that drug resistance studies should be conducted on a wider basis to monitor the emergence of resistance.

Recently, the mechanism of action of several anti-leprosy drugs and the molecular mechanisms of resistance has been established, and facile genetic assays to determine patterns of drug resistance are emerging. Specific missense mutations within the *rpoB*, *folP1*, and the *gyrA* genes of *M. leprae* result in the development of rifampin, dapsone, and ofloxacin resistance, respectively. Based on this information, several PCR-based mutation detection assays have now been developed to detect the presence of these mutations and, therefore, the drug susceptibility of *M. leprae* directly from patient biopsy specimens. PCR-direct DNA sequencing assays have been developed for rifampin, dapsone, and ofloxacin resistance. These assays are the most definitive assays as they generate the actual DNA sequence of the isolate. PCR-single strand conformation polymorphism analysis (PCR-SSCP) and a related assay, PCR-heteroduplex analysis (PCR-HDR) have been developed for detection of rifampin and dapsone resistance, respectively. PCR-line probe assay (LiPA) has been developed for detection of rifampin resistance. All of these assays can be performed within 24 hr and, therefore, provide rapid drug susceptibility data for clinical use and are now available to assess local or global burdens of drug-resistant leprosy. The mechanisms of action of clofazamine and minocycline on *M. leprae* are still largely unknown.

General Applications of PCR in Diagnostics. PCR-based assays are highly sensitive and specific, able to detect small numbers of *M. leprae* in various types of clinical specimens, such as slit skin smear, skin biopsy, and nasal swab specimens, as well as *M. leprae* in the environment, and thus have broad clinical applications and can be important in epidemiological studies. Still, PCR in the leprosy context is confined to the research arena. The ideal PCR for leprosy diagnosis must be simple and short on manipulations, reproducible, highly specific, and sensitive. Meanwhile, many kinds of PCR amplification have been introduced into leprosy research: DNA-PCR, reverse transcription-PCR (RT-PCR), nested PCR, NAOBA amplification, and modern quantitative amplification, real-time PCR. Such sophisticated PCR is difficult to implement for routine diagnosis, but it does provide distinct advantages: for instance, nested PCR is a two-step amplification of DNA that enhances sensitivity and specificity of assays; RT-PCR is an amplification of RNA molecules and is much more sensitive than DNA-PCR according to the multiple copies of RNA in cells, and, in addition, it may be capable of detecting viable bacilli. The use of PCR in aspects of leprosy control is crucial, particularly when definite diagnosis of leprosy is imperative. The challenge now for researchers is to develop procedures that are less demanding and more applicable to routine settings.

Assays for γ-IFN and Other Cytokines. Is This a Realistic Tool in the Context of the New Diagnostics? Serological assays for leprosy identify mainly multibacillary patients with lepromatous disease and are frequently negative in tuberculoid or paucibacillary patients. T-cell responses can be easily measured using DTH skin testing. Another alternative is the assay of γ-IFN and other cytokines in whole blood as a measure of T-cell responses. In general, diluted whole blood cultures are stimulated with mycobacterial antigens for six days and T-cell cytokines measured in culture supernatants quantitated by ELISA. In one study, IFNγ responses to *M. tuberculosis* PPD were measured in the context of a BCG vaccination study conducted by Dr. Dockrell and carried out in parallel in Malawi and the U.K. The IFNγ assay showed the different responses to BCG vaccination in the two settings: IFNγ responses to PPD paralleled the Mantoux skin test response, although there were discordant individuals. Responses to individual recombinant antigens can also be detected, but the assays are not sufficiently sensitive to detect IFNγ responses to single peptides at present. When used with a specific *M. leprae* antigen, these assays could complement or replace skin test responses. The goal would be to develop a simple dipstick assay for IFNγ to complement the detection of anti-PGL-I antibodies. Such assays could also be used to measure other cytokines

such as IL-5, IL-10, or TGFβ whose production may distinguish subjects developing clinical disease from exposed individuals who will remain healthy. Thus, presently, such whole blood assays provide a suitable tool with which to address T-cell responses on a limited immuno-epidemiological scale. However, to be assessed as a diagnostic tool with broader applicability, *M. leprae*-specific T-cell antigens are needed as well as technological simplification of cytokine detection.

New Skin Test Trials. National regulatory control over the manufacture of drugs, skin test antigens and vaccines, and the implementation of clinical trials involving human subjects with these products is necessary to promote and protect public health. Such policies ensure the safety, efficacy, and quality of products. Although rules vary among countries, national regulatory authorities are making great strides to establish, refine, and globalize regulatory requirements for biologicals. There are three major components required to introduce a new drug substance: (1) clinical trials; (2) manufacture; and (3) approval. Clinical studies are regulated globally because of the Nuremberg Code, established in 1947. The Declaration of Helsinki followed to become the first international code to satisfy the moral, ethical, and legal concepts in the conduct of human participant research. The process to establish clinical trials involves many critical steps ranging from identifying the trial location to reporting trial results. Regulation of the manufacturing process is also highly regulated to control for the efficacy, potency, safety, identity, sterility, and stability of the drug substance. Many of the agencies governing Good Clinical Trial Practices (GCP) and Good Manufacturing Practices (GMP) are the same within and between countries, yet some countries are still in the process of establishing these regulations. Compliance is controlled by an extensive, complicated, and lengthy approval process. Firstly, an "Investigational New Drug" (IND) application is submitted, followed by protocols, consent forms, and manufacturing information. Upon approval from local and foreign regulatory agencies, an IND number, Single Project Assurance (SPA) number, and protocol numbers are obtained to perform Phase I/CR II clinical trials.

The group at Colorado State University, U.S.A., has moved over a period of ten years, through this process in order to perform new skin test antigen Phase I and Phase II clinical trials in conjunction with the group at Anandaban Leprosy Hospital, Nepal. The two *M. leprae* native antigens, MLSA-LAM and MLCwA, are injected intradermally at 10 and 1 microgram/ml concentrations. Part A of a Phase II trial (Phase I having been successfully accomplished in the U.S.A.) (10 healthy, unexposed subjects) has been completed; pending safety reviews of the results obtained, parts B and C will proceed shortly. Thus, a template has been established to expedite and simplify the process for the implementation of future generations of skin test antigens or vaccines.

New Diagnostic Antigens. Focused, cooperative, and innovative strategies are being implemented to develop and test new *M. leprae* antigens as potential diagnostic reagents. The native *M. leprae* antigens (MLSA-LAM and MLCwA) that are currently being evaluated in Nepal in Phase II clinical trials are being further fractionated to identify which active protein components confer a specific response for *M. leprae* exposure and infection. In addition, unique *M. leprae* proteins have been identified through comparative analysis of the *M. leprae*, *M. tuberculosis*, and other mycobacterial genome databases. Native subfractions and recombinant proteins are being evaluated by DTH responses in sensitized guinea pigs and IFNγ responses of PBMCs from leprosy patients. Currently, five unique recombinant *M. leprae* proteins and a panel of 58 synthetic peptides (selected based on their ability to bind to HLA class II molecules) from 28 unique proteins are being evaluated in a multi-laboratory exercise for IFNγ responses of leprosy patient PBMCs. It is expected that an analysis of serological (B-cell antibody) and T-cell epitopes from these antigens will result in the development of improved *M. leprae*-specific diagnostic tools.

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