Progress and New Directions in Genetics of Tuberculosis An NHLBI Working Group Report

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Tuberculosis (TB), along with AIDS and malaria, is one of the three major killers among infectious diseases. New approaches to preventing, diagnosing, and curing TB are needed, which depend on a better understanding of *Mycobacterium tuberculosis* and the host. The National Heart, Lung, and Blood Institute convened a working group to develop recommendations for future TB research, including genetic aspects of the disease. The following areas were identified: (1) animal model research to improve understanding of persistence, reactivation, and granulomatous reactions; (2) preclinical studies aimed at shortening treatment of TB; (3) new resources for manipulating and characterizing the *M. tuberculosis* genome, proteome chips for more specific diagnoses, and studies of genes that appear to be essential but whose functions are not known; (4) prospective studies associated with clinical trials in populations with or at risk of TB to advance development of diagnostics and prognostics; (5) new quantitative and bioinformatic approaches to study the interaction between *M. tuberculosis* and the infected host and how this influences the infection process; (6) molecular characterization of *M. tuberculosis* genome diversity and phylogenetic analysis; (7) coordinated studies of human genome scans; (8) genetic epidemiology studies; (9) activities to foster knowledge dissemination, education, and training; and (10) coordination between the National Institutes of Health, the Gates Foundation, the Global Alliance for Tuberculosis Drug Development, and other organizations

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Tuberculosis (TB), one of the earliest recorded human diseases, is still one of the biggest killers among infectious diseases. The international death toll due to its causative agent *Mycobacterium tuberculosis* (*Mtb*) is approximately 1.9 million people each year (1). The same study reported that there are approximately 8.8 million new infections annually, and that one-third of the world's population is infected by this pathogen. In the United States, where the infection rate is low, about 10 to 15 million people are estimated to have latent TB infection (<http://www.cdc.gov/nchstp/tb/pubs/TaskForcePlan/TOC.htm>). Previous review articles in the *AJRCCM* have described various aspects of the disease and its social implications (2–4).

Infection can lead to a potentially fatal outcome or an asymptomatic quiescent or latent state, but one in which latent bacteria can be reactivated to cause disease. Immunocompromised individuals and those subject to the ills of poverty are particularly at risk. The frightening public health problem of TB exists despite the widespread use of the *Mycobacterium bovis* bacillus Calmette-Guérin live attenuated vaccine and several antibiotics. This vaccine is variable in protection and does not prevent reactivation or reinfection (5), and the antibiotics in current use are losing their efficacy because of emerging drug resistance (6, 7). To rationally develop new strategies, we must learn how these bacteria circumvent host defenses to survive in infected human hosts, sometimes for years, and can then be reactivated to cause disease. It is essential to understand why relatively few individuals exposed to *Mtb* do contract the disease (1), because defining genetic determinants and correlates of host resistance/susceptibility to TB can help in the design of more efficient vaccines. It is equally important to understand why some clinical *Mtb* strains are more virulent than others, at least in animal models (8), and how *Mtb* reacts to the host at different stages of infection. Host responses to different *Mtb* clinical strains are also an important area for study. Successful research in these areas will aid in the design of new therapies.

CLINICAL MANIFESTATIONS OF DISEASE

Most TB infections are initiated by the respiratory route of exposure now that milk products are generally pasteurized, at least in the developed world. One study in 1978, before the AIDS epidemic, showed that 85% of new TB cases were pulmonary (9). Thus, the different forms of the disease that are discussed below usually arise from dissemination of the bacilli from the infected lung. TB in many cases follows a general pattern as described by Wallgren (10), who divided progression and resolution of the disease into four stages. In the first stage, dating from 3 to 8 weeks, after *Mtb* is inhaled in aerosols and implants in alveoli, the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the so-called primary or Ghon complex. At this time, conversion to tuberculin reactivity occurs. The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs, including other parts of the lung, and at this time sometimes fatal disease can occur in the form of tuberculous meningitis or miliary (disseminated) TB.

Pleurisy or inflammation of the pleural surfaces can occur during the third stage, lasting 3 to 7 months, causing severe chest pain, but this stage can be delayed for up to 2 years. It is believed that this condition is caused by either hematogenous dissemination or the release of bacteria into the pleural space from subpleural concentrations of bacteria. The free bacteria or their components are believed to interact with sensitized T lymphocytes that are

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attracted and then proliferate and release inflammatory cytokines (11). The last stage, resolution of the primary complex, where the disease does not progress, may take up to 3 years. In this stage, longer developing extrapulmonary lesions—for example, those in bones and joints and frequently presenting as chronic back pain—can appear in some individuals. In addition, other organ systems (e.g., genitourinary, nervous, circulatory) can be affected.

Most humans who are infected with TB do not exhibit progression of the disease. One-third of exposed HIV-negative individuals become infected and, of this number, 3 to 10% develop the disease in their lifetimes, whereas HIV-positive individuals infected with *Mtb* have a 7 to 10% chance of developing TB each year (12). TB in HIV-positive adults, whether resulting from reactivation or new infection, is frequently pulmonary (13), but extrapulmonary TB is often associated with AIDS and has been accepted as one criterion for an AIDS-defining diagnosis; its frequency among patients with AIDS ranges from 25 to 66% (14). It is believed that the host inflammatory response, especially overproduction of the cytokine tumor necrosis factor α (TNF- α), plays a major role in tissue damage associated with active TB (15). In the absence of antibiotic treatment or if *Mtb* resistant to front-line antibiotics is involved in the infection, over 50% of individuals with active TB die (14). This high mortality rate results from widespread destruction of the patient's lungs.

EVENTS IN THE INFECTIOUS PROCESS

In the human host, *Mtb* usually enters the alveolar passages in the form of aerosols, where its initial contact is with resident macrophages and dendritic cells (DCs). The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors (16). On entry into an immunologically naive host macrophage, *Mtb* initially resides in an endocytic vacuole called the phagosome that does not progress into a later endosome. This avoids the normal endocytic maturation cycle observed with phagocytosed particles (i.e., phagosome-lysosome fusion), in which bacteria encounter a hostile environment that includes acid pH, reactive oxygen intermediates, and lysosomal enzymes. Reactive nitrogen intermediates (RNIs) such as nitric oxide (NO) and its derivatives can arise from the activity of inducible NO synthase (iNOS) in numerous cells, including activated macrophages. RNIs are major elements in antimycobacterial activity in the mouse (17). Although iNOS activity or RNIs are not usually observed in human macrophages from cell lines or macrophages derived *in vitro* from blood monocytes of healthy humans, alveolar and tissue macrophages from lungs of patients with TB show high levels of functional iNOS (18). In addition, infected lung tissue from patients with TB shows high levels of nitrotyrosine, a product of NOS activity, as well as elevated levels of iNOS activity (19). It is not known how *Mtb* sometimes avoids the killing activity of macrophages that is observed with other pathogens. However, cell wall components like lipoarabinomannan and secreted proteins like the 19-kD lipoprotein interact with macrophage receptors, and these interactions can modulate the signaling systems used by infected macrophages and DCs to activate the innate immune response (20). For example, the 19-kD protein binds to Toll-like receptor 2, and this interaction results in the repression of the interleukin (IL)-12 and the major histocompatibility complex (MHC) class II presentation pathways (21).

On the basis of much of the above evidence, many researchers favor the possibility that the macrophage has the major role in killing *Mtb* in the human host (22), and one research goal is to identify macrophage effector molecules. In the mouse, these include but are not limited to RNIs; others, some of which may

be under the control of the LRG47 protein (23), remain to be identified. Another hypothesis is that the key cell types responsible for killing *Mtb* during infections are lymphocytes that produce granulysin and perforin, although granulysin seems to be lacking in the mouse and no clear role for perforin in the control of murine TB has been defined. This line of inquiry tends to focus on the strength on human cytotoxic lymphocyte (CTL) responses and how they may be enhanced by vaccination strategies.

The relative ease of working in tissue culture has provided much useful information on *Mtb* entrance and trafficking in the macrophage and on other responses of the infected cells, but much less is known about how the bacterium survives and grows during later stages of infection in the lung. Infected macrophages, through their production of chemokines, attract unactivated monocytes, lymphocytes, and neutrophils (24), none of which kill the bacteria efficiently (22). In some infected people, the disease does not progress past this stage and bacteria are ultimately cleared completely. Other individuals form granulomatous focal lesions composed of macrophage-derived giant cells and lymphocytes. This process is generally an effective means for containing the spread of the bacteria. A strong cell-mediated immune (Th1) response is necessary for granuloma formation, and TNF- α is especially important in this process (25). In the enclosed granuloma, bacilli-loaded macrophages are killed, and this results in the formation of the caseous center that is surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes (26). Although *Mtb* bacilli are postulated to be unable to multiply within this caseous tissue, due to its acidic pH, low availability of oxygen, and the presence of toxic fatty acids, some organisms may remain dormant but alive, for decades. The strength of the host cell-mediated immune response determines whether an infection is arrested here or progresses to the next stages. This enclosed infection is referred to as "latent" TB and can persist throughout a person's life, in an asymptomatic and nontransmissible state. With efficient cell-mediated immunity, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, diabetes mellitus, aging, or other factors, the center of the granuloma can become liquefied in an unknown manner and then serves as a rich medium in which the now-revived bacteria can replicate very rapidly. At this point, viable *Mtb* can escape from the granuloma and spread within the lungs (active pulmonary TB). It is at this stage that *Mtb* can enter alveoli and bronchi of the lung, and the infected individual becomes infectious because the bacilli can be disseminated to others by coughing or expectoration. *Mtb* can also be spread to other tissues via the lymphatic system and the blood (miliary or extrapulmonary TB). When the disease progresses to this point, intensive antibiotic therapy is essential for survival (26).

Little is known about the mechanism(s) of TB reactivation. Some experiments on "persistence" or "latency" have been performed using chronic mouse infection models in which bacterial numbers can be maintained in a steady state, in the absence of disease. Reactivation of bacterial growth and histopathology in these models can be induced by the administration of the iNOS inhibitors N-iminoethyl-L-lysine (27) and aminoguanidine (28). These results and other data strongly suggest that cell-mediated immunity (i.e., IFN- γ induction of iNOS and Th1 cytokines) plays a major role in limiting bacterial growth in infected animals. It is still not certain whether the bacteria in the chronic disease models are actually viable but nongrowing, which would reflect a true latent state, or whether they are growing and dying at the

same rate. An early observation showed that *Mtb* in chronically infected mice is partially susceptible to isoniazid (29), a drug that only is effective against growing *Mtb* (30), providing evidence for both explanations—that is, noncycling persistence and balanced growth and death. Recent experiments now strongly suggest that the bacteria in the chronic disease models are actually viable but nongrowing (31). Moreover, mouse models of acute and chronic TB are different from the human disease in many regards. Other animal models of TB, such as guinea pig, rabbit, and, more recently, monkey infections, are closer in some respects to their human counterpart (32, 33). This is especially true for the formation and development of pulmonary granulomas in human TB infections that show caseation and cavitation (34). Another issue with the mouse “latency and reactivation” experiments is that iNOS plays a major role in these processes. Although iNOS is expressed in tuberculous lesions in humans, as discussed above, there is no direct evidence for a protective role for RNIs in human TB and the role of these compounds in human TB is unclear.

Although we know that strong cell-mediated immune responses are necessary for protection and that CD4⁺ T cells are needed to end the lymphohematogenous stage of early infection in animal models and humans, we still lack critical knowledge regarding the mechanism(s) that govern the induction and maintenance of latent TB infection in humans. Investigations involving persons who have had active versus latent TB infection suggest that latency might be associated with stronger CD8⁺ CTL responses (35). In addition, *Mtb*-specific CD8⁺ T cells preferentially recognize and lyse heavily infected antigen-presenting cells, suggesting a role for these lymphocytes in immunosurveillance (36). This hypothesis is consistent with experiments in mice in which suppression of CD8⁺ T cells with anti-CD8 antisera hastens reactivation of TB in a latency model of infection (37). The antigen-presentation pathways involved in inducing CD8⁺ CTL responses in humans are being actively studied. A currently discussed possibility is that either *Mtb* or its antigens escape from the phagosome into the cytosol and are presented via proteasome–MHC class I pathways (38). A related hypothesis proposes apoptosis-associated cross-priming in which mycobacterial antigens in the cytosol of the infected macrophages are packaged into apoptotic blebs. These structures subsequently fuse with DCs, the primary antigen-presenting cells to naive T cells, so that the antigen contents of the blebs become cytosolic in the DCs and can be presented efficiently (39, 40). Thus, differences in the apoptosis of monocytes and macrophages during initial infection with *Mtb* might determine how well microbial antigens enter into apoptosis-associated cross-priming pathways and might thereby influence whether active or latent disease develops. Also, a role for TNF- α in controlling later stages of human TB infection and latency has been postulated, as elevated levels of this cytokine are found in chronically infected human lung tissue (36), and the use of anti-TNF- α agents like infliximab to treat rheumatoid arthritis and Crohn’s disease is associated with the reactivation of latent TB (41).

Currently, there is little information on how *Mtb* responds to the environment of the lung. There is biochemical evidence that the intermediary metabolism of *Mtb* is different during the course of mouse infections than that observed in bacteria growing in broth culture because fatty acids seem to be a preferred carbon source for *Mtb in vivo* (42–44), but the significance of these apparent changes in metabolism for acute or chronic infection is not known. Recent experiments have shown that RNIs induce a unique pattern of gene expression in *Mtb* (45), and specific *Mtb* genes show different patterns of expression in wild-type as compared with IFN- γ knockout mice (46). This is consis-

tent with *Mtb* in lungs responding to the onset of cell-mediated immunity.

Recent methodologic advances in *Mtb* genetics, such as more efficient allelic replacement and transposon mutagenesis (47, 48), as well as the DNA sequencing and annotation of the *Mtb* H37Rv genome (49) and those of related mycobacteria that have been or are currently being completed by the Sanger Center–Pasteur Institute consortium and by the Institute for Genomic Research (50) have led to a burst of new information on the genetics of *Mtb* virulence. The demonstration that large-sequence polymorphisms are unique events that generate robust phylogeographic bacterial population structures provides a rational framework for understanding the consequences of genetic variation among clinical isolates of *Mtb* (51). Recent scientific advances have demonstrated that over 200 genes and the proteins they encode are necessary for *Mtb* virulence (52, 53). These genetic and molecular approaches have allowed researchers to analyze the genes responsible for differences in virulence that are observed in clinical *Mtb* strains (8) and to characterize the proteins they encode as being responsible for these differences in ability to cause disease (54). New National Institutes of Health (NIH) initiatives to fund a comprehensive mutant analysis of the *Mtb* genome are now underway and approximately 900 transposon mutants have been obtained (NO1 30036, awarded to W. Bishai, Jr.). At the same time, some progress has been made in discovering genetic loci in mice that determine susceptibility/resistance to *Mtb* infection (55–58). Likewise, specific human population cohorts are being analyzed to elucidate the reasons for high susceptibility to TB (59–62). In some cases, correlations have been made with specific human genetic loci (63, 64). A new project to use whole genome scans with multiple single nucleotide polymorphisms (SNPs) to study 1,000 patients with TB and 1,000 control subjects is now underway and should provide valuable information on human genetic loci (A.V. Hill, personal communication, 2005). Thus, genetic tools are now in place for integrating rigorous population genetic analysis of both the pathogen and the host.

FUTURE DIRECTIONS

The National Heart, Lung, and Blood Institute Working Group identified the following areas as important for future research. It is important to stress that the goals of the workshop did not include vaccine development, clinical management, diagnostics, or drug development. This did not reflect a lack of interest. On the contrary, these issues have such high priority that they are being addressed by other working groups and funding mechanisms.

1. Animal model research to illuminate stages of human TB, such as persistence and reactivation, and to improve understanding of specific granulomatous reactions, such as caseation and cavitation, including the following:
 - Conditions in which mice, guinea pigs, rabbits, and monkeys can be used to model each stage and manifestation of human disease (e.g., to gain a better understanding of the granuloma)
 - A better characterized and more uniform set of experiments to be used to delineate TB pathogenesis in diverse experimental animal models. This will be helpful in comparing results from different laboratories.
 - Metabolic characterization of host compartments (e.g., granulomas and cavities) in animal models with respect to Po₂, carbon sources, and other factors relevant to *Mtb*’s metabolism

- Studies to provide a better understanding of host susceptibility to TB, including the genetic and nongenetic aspects (e.g., environmental factors, such as nutritional state of host, age, etc.)
 - Better understanding of host responses to *Mtb* infection at the molecular, cellular, and whole animal level. As an example, studies of suppressive/regulatory pathways of immunity and their genetic control in lungs and central lymphoid organs and the host signaling pathways that are affected by *Mtb* infection.
 - New technologies to study the above issues
2. Preclinical studies to identify chemotherapeutic approaches that may shorten treatment times for active TB. Specific areas are as follows:
 - Development and use of target-specific whole cell assay systems, including human cells and cell–cell interactions, suitable for functional or chemical genomics including screening for chemical inhibitors. For these studies, hypomorphs and hypermorphs, which are defined as strains expressing each gene at subnormal or supranormal levels, will be valuable tools for assessing the specificity of chemical inhibitors of the gene products, according to the increased susceptibility or resistance of the mutant strain compared with the wild-type. Also, global gene expression profiling, secondary promoter–reporter approaches to identify inhibitors of gene products of primary interest via the response of genes whose transcription is affected secondarily by disruption of the primary gene.
 - Resources for chemical biology, including support to set up chemistry core facilities and high-throughput screening facilities and to purchase diversity collections and focused, secondary chemical libraries
 - Support for multidisciplinary groups whose expertise includes microbiology, immunology, genetics, biochemistry, structural biology, chemistry, medicinal chemistry and/or pharmacology, and quantitative or systems approaches to biology
 3. New resources for manipulating and characterizing the *Mtb* genome, including a comprehensive program to inactivate every *Mtb* open reading frame. This will complement existing work in progress. The production of bacterial hypomorphic and hypermorphic alleleles for each *Mtb* gene is a valuable goal as noted above. Advanced proteomic techniques to produce complete proteome chips that could be used for earlier and more specific diagnoses and monitoring of prophylactic and therapeutic interventions. Specific areas are as follows:
 - Regulated promoters for titratable, *in vivo*–active conditional gene activation and inactivation
 - Phage bank and distribution resource for constructs to disrupt all *Mtb* genes in different *Mtb* clinical and laboratory strains
 - Phage bank and distribution resource for a panel of hypomorphic and hypermorphic alleles of *Mtb* genes. Phage bank and distribution resource for unmarked biological constructs for multiple deletions.
 - Studies of the functions of bacterial genes that appear to be essential for virulence but whose function is unknown, as this will lead to an improved understanding of *Mtb* biology in the context of the infectious process
 4. Prospective studies associated with vaccine or other clinical trials in populations with or at risk of TB to advance the development of diagnostics and prognostics, including the following:
 - New and improved methods of target validation *in vivo*, including humans (e.g., *Mtb* gene expression analysis at a global level *in vivo* at low bacterial numbers). These studies will be very important for a better understanding of latency, persistence, and reactivation.
 - Serology at a pan-proteomic level—for example, to allow tests of specific antibodies that might help reveal what proteins are expressed by *Mtb* at what stage in which hosts, and to see if reactivation can be detected earlier via the host antibody response than can be achieved by conventional methods such as chest X-rays and sputum analysis
 - Assessment of subjects' medical status with respect to diabetes, atherosclerosis, hypertension, cardiovascular disease, obesity, and “metabolic syndrome” as possible risk factors for reactivation and predictors for response to vaccination
 - Studies to identify sterilization of infection versus latent infection
 - Studies to determine who should be treated for latent infection. The recently suggested promise of DNA vaccines for the prevention of reactivation and reinfection of *Mtb* during chemotherapy (65) illustrates an important connection with this area of development.
 - Studies to predict reactivation and prevent transmission
 - Identification of genetic and immune markers for human protection and susceptibility (to better understand why only 10% of infected individuals contract TB)
 - Rapid spot funding to investigate any of the above, as well as mechanistic questions, in the framework of clinical studies, where the new issues arise after the clinical study was designed
 5. New quantitative and bioinformatics approaches to study *Mtb*–host interactions and their effect on infection outcome, including the following:
 - Combined transcriptome analyses of *Mtb* and infected macrophages and granulomas, using DNA arrays and other technologies (e.g., laser-capture methodology), as well as the development of post-transcriptome analyses. These approaches could help in the identification of new regulatory networks that may help explain pathogenic mechanisms and lead to new therapies.
 - Bioinformatic modeling to understand data obtained with DNA arrays and other global searches and its use to predict outcomes
 6. Characterization and phylogenetic analysis of *Mtb* genome diversity to provide novel approaches to understanding gene function and host–pathogen interactions. Specific areas are as follows:
 - Complete sequencing of five additional strains representative of geographically distributed phylogeny will add considerable insight to sequences obtained to date.

- Development of high-throughput SNP typing based on five whole genomes and segment sequencing of 100 more strains will be an invaluable tool for large-scale comparative genomic studies.
 - Compilation of a minimum panel of strains that are quantitatively representative of genetic diversity among clinical isolates (aside from drug resistance) as an aid to testing new diagnostics, vaccines, and chemotherapeutics
 - Integration of these improved tools for bacterial population analysis of *Mtb* with ongoing studies of human genetic variability have the potential to fundamentally alter our understanding of the relationship between host and pathogens.
7. Coordinated studies of human genome scans to allow multiple research groups to cooperate to obtain large numbers of samples that would be used for surveying diverse human populations for susceptibility to TB
 8. Genetic epidemiology studies to predict how susceptibility and resistance genes in populations affect the overall prevalence of disease and trends for epidemics
 9. Activities to foster knowledge dissemination and education, including the following:
 - Workshops for standardization of definitions, reagents, assays, protocols, and mouse strains for vaccine development, and other preclinical and clinical studies
 - Sponsorship of meetings to review new technical opportunities, to plan or promote awareness of clinical trials, to coordinate human genome scans
 - Training programs in microbial physiology
 - Technical transfer to endemic areas
 - Future meetings on topics such as latency—what is known, how to model it, how to use human data, limitations, possible drugs, and so forth
 10. Coordination between private and public funding agencies should be fostered. However, the product development efforts that use coordinated and focused management structures that are being funded by the NIH, Bill and Melinda Gates Foundation, and others should complement and not compete with existing discovery-based research. The translational activities of organizations such as the Global Alliance for Tuberculosis Drug Development, Aeras Tuberculosis Vaccine Foundation, and the Foundation for Innovative New Diagnostics provide an unprecedented opportunity for synergy between basic and applied research. Coordination would include advisory resources to help guide individual investigators or consortia along a path from target validation to hit identification to lead development to drug development to clinical trials, with each of the relevant agencies contributing expertise at the appropriate point and cooperating to hand off a given project to other agencies as appropriate.

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