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Molecular approaches to tuberculosis

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Introduction

Tuberculosis, a predominant disease in the nineteenth century, remains one of the most life-threatening diseases at the beginning of the new millennium. It causes two million deaths per year. The incidence of the disease has been always high in developing countries. However, its re-emergence in industrialized countries is a major public health problem and was completely unexpected by the general public, researchers, doctors and the authorities. Nobody could have predicted that tuberculosis would strike again, despite the continuous decline in the incidence of tuberculosis between 1800 and 1950, the development of effective antituberculosis drugs, and improvements in public health and living conditions. What went wrong? Or, perhaps, why did things go wrong? And, of course, what can we do to control tuberculosis now and in future?

Hopefully, we now know more about tuberculosis than in the nineteenth century, when the fresh air of the mountains was the only prescription available, as Thomas Mann beautifully described in *The Magic Mountain.* In 1993 the World Health Organization declared that tuberculosis was a global health emergency. Since this time, considerable effort has improved our knowledge on the biology of these bacilli and their interactions with humans. Studies on this pathogen revealed new pathways involved in antigen presentation and T-cell recognition. Genetic tools were used to identify virulence genes. The availability of several mycobacterial genome sequences might elucidate host–pathogen interactions. Thus, knowledge of the human genome sequence will provide new tools for intervention.

The Juan March Workshop on Molecular Approaches to Tuberculosis (Madrid, 11–13 December 2000) brought together many researchers from around the world to discuss the current understanding of tuberculosis from a molecular point of view, and the perspectives for tackling this disease. The genome, genetic analysis, the ultrastructure of the cell wall, mechanisms of drug resistance, strategies for developing new drugs, strategies for survival within infected cells, relevant mycobacterial antigens, virulence determinants, induced alterations in cytokine circuits, host–pathogen interactions and vaccine development were discussed.

Molecular knowledge of a bacterium and its host is essential for the control of a disease. However, to consider a bacterium just a compendium of molecules and to consider a disease only in terms of molecular interactions is too simplistic and not totally realistic. Douglas Young (Imperial College, London, UK) emphasized the social and economical implications of tuberculosis, a highly transmissible disease. Curing one patient can prevent many other people from getting infected. An infected individual's particular susceptibility (owing to genetic and nutritional causes, whether they have been vaccinated and their immune status) will determine whether they will develop the disease. As one third of the world's population is believed to be infected with the tubercle bacillus, the risk of developing an active disease after infection is a crucial issue. Our immediate objective is to cure patients with active tuberculosis and the next stage will be to reduce the probability of developing the disease after infection, for example, using a vaccine. Social and economic factors are as important as molecular approaches, and the final solution will include all of these aspects.

Genetics and genomes

Stewart Cole (Institut Pasteur, Paris, France) summarized the main features of the *Mycobacterium tuberculosis* H37Rv genome. It contains approximately 4000 putative genes (of which 60% have a predicted function) that provide *M. tuberculosis* with a wide range of metabolic pathways, including those for aerobic, microanaerobic and anaerobic metabolism (Cole *et al.*, 1998). Lipid metabolism involves many genes, as predicted by the complexity and diversity of lipid content in the mycobacterial cell wall. *M. tuberculosis* has a high number of regulatory genes, indicating that it must be highly adaptable to changing

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conditions, consistent with the environmental origins of the mycobacteria, which only became a human pathogen about 15 000 years ago (Sreevatsan *et al*., 1997). Many repetitive genes, mostly of unknown function, such as members of the PE and PPE families, may be related to antigenic variation, conferring plasticity to both the genome and the bacillus.

Other mycobacterial genomes (*M. leprae, M. tuberculosis* CDC1551 and 210, *M. bovis* AF2122/97 and BGC-Pasteur, *M. smegmatis*, *M. avium* and *M. paratuberculosis*) have now been either completely sequenced or will be soon. Genome comparisons may expand our knowledge on the molecular bases of pathogenicity, virulence and host specificity, and evolutionary aspects may also be revealed. Libraries of BAC-arrays (Gordon *et al*., 1999; Brosch *et al*., 2000) and DNA microarrays (Behr *et al*., 1999) have been used for genome comparisons. To date, five deletions (RvD1-RvD5) have been detected in the genome of *M. tuberculosis* H37Rv in comparison with *M. bovis* genome, and at least 16 deletions (RD1-RD16) have been detected in the chromosome of the Pasteur substrain of the vaccine strain *M. bovis* BCG. The distribution of these deletions among species of the *M. tuberculosis* complex has established new evolutionary relationships, suggesting that *M. tuberculosis, M africanum, M. microti, M. bovis* and *M. bovis* BCG appeared sequentially from a common ancestor. This rules out the hypothesis of human tuberculosis having a bovine origin.

M. leprae was long thought to have been mistakenly included in the genus *Mycobacterium*, because of its low G + C content (58%). Stewart Cole showed that the 3.2 Mb genome of *M. leprae* (Cole *et al.,* 2001) contains around 1600 genes, most of which are similar to *M. tuberculosis* genes. These 1600 genes can be considered as the minimal gene content for pathogenicity. *M. leprae* has a large number of pseudogenes, i.e. genes with premature stop codons or frameshift mutations in comparison with the *M. tuberculosis* counterparts. The scarcity of genes prevents *M. leprae* from using many metabolic pathways, especially those involved in catabolism and NADH utilization. This explains why this species has become an obligate intracellular organism and cannot grow on culture media.

Many research projects have benefited from having the *M. tuberculosis* genome readily available on the tip of your keyboard, e.g. L. E. Quadri (Cornell University, New York, NY, USA) used genome information to describe the first steps of the mycobactin biosynthesis, the siderophores produced by *M. tuberculosis* (Quadri, 2000). For pathogens living and infecting mammalian hosts (such as *M. tuberculosis*), iron becomes an extremely precious metal (how many alchemists would have dreamed the same!) and siderophores are essential compounds that help the bacteria to get this nutrient from the mammalian iron-binding proteins. The *M. tuberculosis* genome project revealed a 25 kb cluster encoding the enzymes involved in the biosynthesis of the salicyl-capped peptide-polyketide scaffold of mycobactins. Some of these enzymes have been successfully expressed in *M. smegmatis*, and the salicylation and phosphopantetheinylation reactions have been characterized *in vitro.* Mycobactin biosynthesis is an attractive target for the development of new drugs or the construction of new vaccines because mycobactins are essential for *M. tuberculosis* growth inside macrophages (De Voss *et al.,* 2000).

New tools and techniques for analysing mycobacterial genomes

The choice of genetic tools for analysing gene function is probably as important as having access to complete genome sequences. In recent years, there has been much progress in the development of tools such as plasmid and phage vectors, transposons, reporter genes and strategies for gene inactivation and mutagenesis (Hatfull and Jacobs, 2000).

Currently, the aim of most genetic studies on mycobacteria is to find virulence genes (this was probably one of the objectives of the genome project itself), and the genetic tools developed in the last 10 years are being used to reach this goal. C. Guilhot (Institut Pasteur, Paris, France) described the use of signature-tagged transposon mutagenesis (a technology successfully used in the study of other human pathogens; Hensel *et al*., 1995) to generate *M. tuberculosis* mutants and the selection of mutants with growth attenuated in mice during the first 3 weeks of infection (Camacho *et al*., 1999). Not surprisingly, in most of the mutants the transposon was inserted into genes involved in lipid metabolism, emphasizing the importance of this kind of molecule for mycobacterial pathogenesis. Four of these mutations affected a 50 kb region of the chromosome that contains polyketide synthase and the transporter genes involved in the biosynthesis and transport of phtiocerol dimycoserosate (DIM), a lipid found in the outer layer of the mycobacterial envelope. As well as being attenuated, these mutants were more permeable to hydrophobic compounds, such as chenodeoxycholate, and more sensitive to detergents (Camacho *et al*., 2001). These findings indicate that extractable lipids are important for maintaining the permeability of the barrier and for bacterial virulence, perhaps by modulating the host's immune response.

Mycobacteriophages have played a major role in the development of mycobacterial genetics. They were the first system available for transforming mycobacteria. Many different mycobacteriophages have now been isolated and sequenced, a large number of phage-derived vectors have

been constructed and many clinical and research techniques rely on them. In addition, as Bill Jacobs reported, it seems that new isolates can be isolated very simply, for example, from soil samples. Manipulated mycobacteriophages containing, for example, the luciferase reporter gene, are a rapid, simple and reliable method for assessing drug resistance in mycobacteria. Specialized transducing phages can be used as an alternative strategy to construct knockout mutants in mycobacteria.

Genetics of drug resistance

The continuous development of genetic tools should enable us to address the complex questions surrounding mycobacterial interactions with humans. One of these problems is drug resistance, because only a limited number of drugs are active against mycobacteria.

The catalase–peroxidase enzyme, KatG, activates the pro-drug isoniazid (Zhang *et al*., 1992). Most isoniazid-resistant *M. tuberculosis* clinical isolates carry mutations or deletions in the *katG* gene (some studies have shown that these mutations lead to decreased virulence in animal models; Li *et al*., 1998). However, some isolates were found to be resistant to both isoniazid and ethionamide (another first-line antituberculous drug), even though patients had not been treated with ethionamide. This suggested that isoniazid and ethionamide might share a common target. Bill Jacobs (Albert Einstein College of Medicine, Bronx, NY, USA) reported the identification of this target in *M. smegmatis* as the product of the *inhA* gene, an enzyme similar to NADH-specific enoyl ACP reductase (Banerjee *et al*., 1994), indicating that KatG-activated isoniazid probably reacts with NADH to form adduct which binds and inactivates InhA. The inactivated InhA then blocks the type II fatty acid synthase (FASII), impairing fatty acid biosynthesis, and resulting in the accumulation of the saturated fatty acids produced by type I FAS (the other mycobacterial fatty acid synthase), which must be lethal for *M. smegmatis*. Furthermore, the inactivation of a thermosensitive InhA enzyme in the absence of isoniazid also makes *M. smegmatis* cells non-viable (Vilcheze *et al*., 2000). Although *M. tuberculosis* also has an *inhA* gene, other targets for isoniazid have been proposed in this organism, such as the product of the *kasA* gene and an acyl carrier protein (both also involved in type II FAS) (Mdluli *et al*., 1996; 1998). As most isoniazid-resistant *M. tuberculosis* isolates have alterations in KatG rather than in the potential targets, a further study of the target(s) for isoniazid is an attractive issue, because it could lead to the development of new drugs that targeting the same enzymes would circumvent KatG activation.

To overcome the increase of drug resistance in *M. tuberculosis*, one of the most pressing needs is the discovery of new antimycobacterial agents. Two recently developed techniques have facilitated the synthesis and screening of new compounds: combinatorial chemistry and DNA microarrays. Ethambutol is a first-line antituberculosis drug; it affects the biosynthesis of both lipoarabinomannan and arabinogalactan by inhibiting membrane-associated arabinosyltransferases, causing mycolic acids to accumulate and cell death (Telenti et al., 1997; Ramaswamy *et al*., 2000). Mutations in the *embCAB* genes, which encode arabinosyltransferases, have been associated with ethambutol resistance. Cliff Barry (National Institute of Health, Rockville, MD, USA) used DNA microarrays to identify ethambutol-induced genes and their promoters were fused to luciferase gene (Barry *et al.,* 2000a). This construction was introduced into *M. tuberculosis* and used to screen a library of ethambutol analogues synthesized through combinatorial chemistry (the split and pool technique) for compounds that produced bioluminescence. This identified new ethambutol derivatives that are currently being evaluated for their activity against *M. tuberculosis*, both *in vitro* and *in vivo* in animal models, and also for their activity against ethambutol-resistant *M. tuberculosis* isolates. Similar approaches are being carried out with other molecules (i.e. isoniazid); therefore the new generation of antituberculosis compounds look promising for the treatment of this disease (Barry *et al.,* 2000b).

Antibiotic resistance levels and fitness

Erik Böttger (Universität Zürich, Switzerland) described mycobacterial drug resistance, levels of resistance and fitness and their implications for treatment. Firstly, mycobacterial resistance is produced almost exclusively by chromosomal mutations rather than by the acquisition of drug inactivation mechanisms through transmissible plasmids or transposons. Multidrug-resistant isolates appear by sequentially accumulating mutations in individual genes. Unfortunately, the mutations identified in resistant *M. tuberculosis* strains isolated from patients do not seem to carry any detectable cost in terms of bacterial fitness (Böttger *et al*., 1998), which implies that drug-resistant isolates do not have a diminished transmissibility and will persist in the community equally as well as the drug-susceptible ones. Secondly, drug susceptibility in mycobacteria is mostly determined for a single concentration of a given drug. Therefore, the diagnostic of resistance/susceptibility refers to this internationally accepted cut-off concentration. However, this method cannot detect different levels of resistance, which reflect the heterogeneity of the underlying mutations in, for example, streptomycin- or ethambutol-resistant isolates. This range of resistance levels may explain why more than one third of cases of tuberculosis caused by drug-resistant strains can be cured by standard short-term chemotherapy

(Espinal *et al*., 2000). Current treatments do not differentiate between resistant strains harbouring different mutations (and probably exhibiting different levels of resistance). Possibly, re-examining the *true* resistance levels and administering treatment accordingly may prevent new drug resistant isolates from arising and progressively reduce the number of existing ones.

Molecular epidemiology of multidrug-resistant tuberculosis

Carlos Martín (Universidad de Zaragoza, Spain) presented data showing the alarming increase in multidrug-resistant tuberculosis (MDR-TB) in some geographical regions (Espinal *et al*., 2001) and showed that very little is generally known about the genetic characteristics of these strains. In the USA, hospital- and prison-based outbreaks of MDR-TB have been described in human immunodeficiency virus (HIV)-positive patients; some of these strains have been well characterized at molecular level, for example the 'W' strain that belongs to the Beijing family (Bifani *et al*., 1996). MDR-TB outbreaks caused by similar strains have recently been described in western and eastern European countries, and are particularly common in prisons (Portaels *et al*., 1999).

In Spain most MDR-TB cases are secondary to treatment and most MDR *M. tuberculosis* strains were not further transmitted to other patients. However, one particular strain of *M. bovis* (the 'B' strain) has been isolated in more than 100 cases of MDR-TB, mostly in HIV-positive patients (Rivero *et al*., 2001). Compared with other *M. bovis* strains, the 'B' strain presents many pleiotropic differences, including smaller size of the bacilli and slower growth, suggesting altered regulatory processes. Genetic analysis of the 'B' strain revealed that one of the two copies of the insertion sequence IS*6110,* is inserted upstream of the global regulator *phoP*, possibly influencing its expression. Recently, evidence that *phoP* is involved in *M. tuberculosis* virulence has been provided (Pérez *et al*., 2001).

Molecular epidemiology methods have been used to determine whether outbreaks of MDR-TB are caused by particular strains with unknown selective advantages, as in the case of the genotype family strains 'Beijing-W' in the USA or M. bovis 'B' in Spain. These findings suggest that particular *M. tuberculosis* complex MDR strains are better adapted for MDR-TB transmission than others.

The mycobacterial cell wall

The biochemically and structurally complex mycobacterial cell wall has always been one of the major obstacles for studying mycobacteria and for the successful use of antituberculosis drugs. However, important advances have recently been made towards understanding the genetic basis of the biosynthesis of cell wall components and of the biochemical pathways leading to their assembly. This has led both geneticists and biochemists to set their hopes on using the cell wall to develop new drugs and vaccines.

Patrick Brennan (Colorado State University, Fort Collins, CO, USA) reviewed how our understanding of the mycobacterial cell wall has progressed. Although the cell wall constituents and the way in which they are linked have been generally accepted for many years, the three-dimensional structure of the cell wall has changed considerably in recent years. In 1982, the classic Minnikin model presented the mycolic acids arranged in a monolayer outside of the cell wall skeleton and that their hydrocarbon chains were perpendicular to the cytoplasmic membrane, therefore constituting a highly hydrophobic barrier, analogous to the outer membrane of gram-negative bacteria. Underneath the mycolic acid layer, arabinogalactan (to which mycolic acids are covalently bound) and peptidoglycan formed horizontal layers, parallel to the cytoplasmic membrane. This model has been successively modified to incorporate new cell wall constituents and structural features as they were discovered. Recently, biochemical and electron microscopy data led to the proposal of a new model in which both arabinogalactan and peptidoglycan are arranged into short vertical helical strands, perpendicular to the cytoplasmic membrane (Dmitriev *et al*., 2000). The new model considers the actual dimensions of cell wall components and the links between them. It predicts that mycolic acids are tightly packed, which may explain the strong hydrophobicity of the mycobacterial cell wall and the intrinsic resistance to antimicrobial agents. The low permeability of the mycobacterial cell wall may also reflect a low abundance of porins, which are probably species specific (Kartmann *et al*., 1999).

Apart from arabinogalactan, in *M. tuberculosis* mycolic acids are covalently bound to trehalose, forming the trehalose dimycolate, a glycolipid involved with the cording of the bacilli, a phenomenon that has been directly related with virulence. Bill Jacobs described the identification of the *M. tuberculosis pcaA* gene, which is involved in the cyclopropanation of mycolic acids. Disruption of *pcaA* causes alterations in cording, colony morphology and cyclopropanation of alpha mycolates, and affects persistence and virulence (Glickman *et al*., 2000). As glycolipids can be presented to the immune system by CD1 antigens, alterations in their structure will probably affect the interactions with T-cells.

Phagocytosis, a cell inside a cell

For bacteria, the cell wall delimits its interactions with the

environment. In mycobacteria, phagosomes are the immediate environment inside infected cells. Therefore, mycobacterial cell wall components make contact with the cells they invade and activate many processes (such as cytokine production and T-cell proliferation) by interacting with host cell receptors.

Several receptors seem to be involved in internalizing mycobacteria. Isabelle Maridonneau-Parini (Institut de Pharmacologie et de Biologie Structurale, Toulouse, France) explained how neutrophils, for example, can phagocyte *Mycobacterium kansasii* in non-opsonic conditions (Peyron *et al*., 2000). This process is mediated by complement receptor 3 (CR3), which is associated with glycosylphosphatidylinositol (GPI)-anchored proteins. GPI-anchored proteins are located in discrete domains of the cytoplasmic membrane, called rafts, which are rich in cholesterol. The phagocytosis of *M. kansasii* by neutrophils is inhibited both by reducing the concentration of cholesterol in the cells and following treatment with phosphatidylinositol phospholipase C.

Both Alan Aderem (University of Washington, Seattle, WA, USA) and Robert Modlin (UCLA School of Medicine, Los Angeles, CA, USA) elucidated the role of Toll-like receptors in the phagocytosis of mycobacteria by macrophages and the simultaneous process of secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) (Underhill *et al.,* 1999; Krutzik *et al*., 2001). Mammalian cells contain Toll receptor homologues, which mediate the immune response against fungal infections in insects. In macrophages, these receptors are recruited by phagosomes, in which they identify internalized bacteria and act as a surveillance system. Human Toll-like receptor 2 (TLR2) specifically recognizes cell wall components from yeast and gram-positive bacteria (whereas TLR4 recognizes components of gram-negative bacteria). In the case of *M. tuberculosis*, TLR2 (and to a lesser extent other receptors such as TLR6) recognizes several cell wall components such as the mycolylarabinogalactan–peptidoglycan complex and the 19 kDa lipoprotein. This interaction (in which the fatty acid moiety of the 19 kDa lipoprotein seems to be essential) triggers a signalling pathway that eventually activates the expression of nuclear factor (NF)-κB, which is known to activate the production of several pro-inflammatory cytokines, including interleukin 12 (IL-12) and TNF-α. Finally, IL-12 generates a T-helper type 1 lymphocyte response, which eliminates intracellular mycobacteria.

Robert Modlin also showed how infected macrophages display direct antimicrobial mechanisms (Thoma-Uszynski *et al.,* 2001). The activation of TLR2 by microbial lipoproteins activates the expression of the inducible nitric oxide synthase (iNOS) in murine macrophages. This in turn generates the bactericidal molecule, NO, that inhibits the growth of intracellular mycobacteria. However, in human macrophages the induction of iNOS is not observed. This indicates that human macrophages have an NO-independent mechanism of microbicidal activity. Similarly, TNF-α and interferon gamma (IFN-γ) stimulation induces NO production in murine macrophages but not in human macrophages. However, these findings do not exclude the possibility that NO plays a role in alveolar macrophages, the first cells involved in the infectious process (Rich *et al*., 1997).

Alan Aderem explained phagocytosis in more detail (Aderem and Underhill, 1999). Once bacteria are bound to the cell receptors, actin starts polymerizing and engulfs them. Many host proteins are involved in phagocytosis and their selective inactivation blocks the internalization at different stages. For example, the inactivation of anphiphysin completely blocks phagocytosis but does not affect the release of inflammatory factors. However, if the GTPase dynamin is blocked, phagocytosis begins but stops when the bacterium is half internalized.

Cholesterol and mycobacterial survival

One of the key features of mycobacterial survival within macrophages is their ability to arrest intracellular trafficking, which allows bacteria to replicate and survive in the host. Host proteins control trafficking, but live mycobacteria can alter this process to ensure their survival. In phagosomes containing live mycobacteria, a 50 kDa protein was identified, but this protein was not detected in phagosomes containing heat-killed mycobacteria (Ferrari *et al*., 1999). This protein was named TACO because it contains five tryptophane aspartate repeats and is localized in the cortical microtubule network of uninfected macrophages (tryptophane aspartate-containing coat protein). TACO rapidly accumulates at the site of bacterial uptake, and in phagosomes containing live mycobacteria remains at this site for prolonged periods of time. However, when heat-killed mycobacteria are used to infect the macrophages, TACO dissociates from the phagosomes within a few hours of infection, indicating that live mycobacteria are responsible for retaining TACO covering the phagosomes. Mycobacteria are rapidly destroyed in cells lacking TACO, confirming the protective role of TACO for mycobacteria. John Gatfield (Basel Institut for Immunology, Basel, Switzerland) reported that the association of TACO to the membranes is cholesterol dependent, because when phagosomes are treated with the cholesterol sequestering compound digitonin, TACO is separated from the membrane fractions (Gatfield and Pieters, 2000). Interestingly, depletion of cholesterol from macrophages resulted in a reduction of mycobacterial uptake (consistent with Isabelle Maridonneau-Parini's observations in neutrophils) although this did not affect

the uptake of other bacterial pathogens. These findings show that cholesterol is essential for mycobacteria pathogenesis because it facilitates the entry of mycobacteria into macrophages and mediates the association of TACO to mycobacterial-containing phagosomes, which protect mycobacteria from lysosome degradation.

To induce or not to induce IFN-γ production

Following *M. tuberculosis* infection, the balance between the survival and growth of the organism and the kinetics and magnitude of the host immune response determine the outcome of the disease. The immune response normally involves a large number of interactions between cells, usually mediated by the production and detection of several cytokines. Here, IFN-γ seems to play a central role.

M. tuberculosis isolates differ considerably in terms of their growth rate, virulence and ability to stimulate cytokine production (Manca *et al.*, 1999). Similarly, the responsiveness of the immune system may differ from individual to individual (see discussion by Tom Ottenhoff). As a result, a number of situations may occur; 90% of individuals infected will never develop an active disease whereas in the remaining 10% the disease will have different degrees of severity. Gilla Kaplan (The Rockefeller University, New York, NY, USA) focused on the differences between the bacilli and reported how mice infected with the more virulent strains fail to trigger T-cell proliferation and IFN-γ production. In contrast, less virulent strains induce a rapid production of both TNF-α and IL-12. Consequently, antigen presentation, the T-cell response and IFN-γ production occur rapidly. The study of cell fractions of *M. tuberculosis* isolates with different levels of virulence led to the identification of the polar and apolar lipids responsible for the activation of cytokine production.

Host cytokine circuits may therefore be considered to be a network, so it is not surprising that, after infection, stimuli transmitted through separate pathways may lead to contradictory effects. Zhara Toossi (Case Western Reserve University, Cleveland, OH, USA) demonstrated that *M. tuberculosis* can alter cytokine circuits in many different ways, leading to, paradoxically, either a reduction or an stimulation of IFN-γ production.

In infected macrophages, the tubercle bacillus greatly induces the expression of transforming growth factor beta (TGF-β). TGF-β deactivates infected macrophages, suppressing the T-cell response and consequently reducing the levels of IFN-γ messenger RNA that can easily be detected by real-time reverse transcription–polymerase chain reaction (RT–PCR). Modulation of TGF-β by use of LAP (latency-associated peptide) reduces TGF-β activity (Wilkinson *et al.,* 2000). Consequently, the proliferation of T cells is increased and the levels of IFN-γ return to normal. Therefore, mycobacteria induce the expression of TGF-β, which partially neutralizes the host immune response and contributes to the growth and survival of mycobacteria in the lungs.

Conversely, the expression levels of several genes are also altered within the mycobacterial cells upon infection, and cytokines may increase mycobacterial metabolism and gene expression. In macrophages, the induction of expression of the mycobacterial antigen 85B, an enzyme involved in cell wall biosynthesis, increases the presentation of this antigen to the immune system. Consequently, T cells activate the production of IFN-γ, which in turn activates TNF-α production by infected macrophages. The addition of external TNF-α further induces the expression of antigen 85B in mycobacterial cells and the neutralization of TNF-α reduces the expression of antigen 85B (Wilkinson *et al.,* 2001).

Adapt or die: strategies for survival

The survival of mycobacteria within macrophages depends on their ability to respond to and modify the environment. David Russell (Cornell University, Ithaca, NY, USA) illustrated several strategies used by *M. tuberculosis* in its adaptive response to the hostile and changing conditions encountered within macrophages.

Fatty acids are abundant in mammalian tissues and are metabolized by intracellular bacteria through the β-oxidation cycle and the glyoxylate shunt, producing energy and intermediates for other processes such as gluconeogenesis. Recent studies have revealed that in *M. tuberculosis* the expression of *icl* gene, encoding isocitrate lyase, a glyoxylate shunt enzyme, is upregulated following the infection of macrophages (McKinney *et al.*, 2000). The expression of *icl* is also induced by palmitate. An *icl* knockout mutant of *M. tuberculosis* showed no difference in growth during the acute phase of infection, in comparison with wild type. However, the mutant was eliminated progressively as infection proceeded. The mutant also had attenuated virulence. These data indicate that isocitrate lyase activity is essential for establishing a persistent infection.

The mycobacterial phagosomes contain surface-derived MHC class II molecules during the early stages and these molecules tend to disappear as the phagosomes mature. When the macrophages are activated by IFN-γ, the phagosome rapidly acquires MHC class II molecules, which effectively present mycobacterial antigens and elicit the immune response (Ullrich *et al.*, 2000). However, this process can be modified by pathogenic mycobacteria, which allows them to persist in the host cells. Pathogenic mycobacteria produce a large quantity of cell wall lipids that are released into the host cell cytoplasm and into the external intercellular milieu. These lipids

prevent the macrophage from being activated by IFN-γ and promote the formation of the granuloma, which prevents the lymphocytes from accessing the site of infection (Beatty *et al.,* 2000).

Cellular immunity against mycobacteria

It is generally accepted that the antimycobacterial immune response mainly consists of a cellular response. $CD4^+$ $CD8^+$ αβ T cells typically recognize MHC-restricted peptidic antigens. However, cellular immunity against mycobacteria also involves other subsets of T lymphocytes. Marc Boneville (INSERM, Nantes, France) described the role of the γδ double-negative T cells, which are the predominant population in peripheral blood cells in humans and express the Vγ9 and Vδ2 T-cell receptors. Nanomolar quantities of several non-peptide phosphorylated compounds (for example 3-formyl 1-butyl-pyrophosphate, produced by mycobacteria) can selectively activate γδ T cells (Belmant *et al*., 2000). This activation is not MHC restricted but it involves direct interactions with the γδ T cells. Subsequently, γδ T cells activated through the granule exocytosis pathway, release molecules such as perforin that kill mycobacterium-infected macrophages. As it does not require antigen processing and presentation, the contribution of γδ T cells to the immune protection against tuberculosis probably occurs during the early phases of infection (Dieli *et al*., 2000).

Mycobacterial glycolipid antigens can be presented to T lymphocytes by any of the five CD1 isoforms, a kind of non-classic MHC molecule. Group I CD1 molecules are expressed by professional antigen-presenting cells and, upon infection, present glycolipid antigens that activate αβ or γδ T cells. These activated cells then lyse the infected antigen-presenting cells and secrete cytokines to control the microbial infection. Group II CD1 antigens present similar ligands, which are recognized by natural killer (NK) T cells.

Dendritic cells are also infected with *M. tuberculosis*, as reported by Joe Colston (National Institute for Medical Research, London, UK) (Tascon *et al*., 2000). Immature forms of these cells are present in peripheral tissues (such as airway epithelium and the lungs) and contain low levels of MHC class II molecules and co-stimulatory molecules. However, upon infection they become activated and maturate. The mature forms then upregulate the production of several cytokines (for example TNF-α, IL-1β, IL-6, IL-12) and other stimulatory factors. Dendritic cells migrate to lymph nodes in which they will efficiently present antigens to trigger the specific T-cell response. Interestingly, *M. tuberculosis*-infected dendritic cells are able to immunize mice against tuberculosis, as demonstrated by the high levels of IL-2 and IFN-γ produced in response to specific antigen stimulation.

As the immune response progresses, a large number of cell interactions take place in the lung granuloma, in which infected macrophages and different types of lymphocytes coexist for a period of time. Mercedes Gonzalez-Juarrero (Colorado State University, Fort Collins, CO, USA) studied the temporal and spatial arrangements of the different cell types in a granuloma (Gonzalez-Juarrero *et al*., 2001). During the early infection (less than 40 days), the number of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells increases considerably in the lungs. Most cells within granulomas are $CD4^+$. After longer periods of time (>100 days), $CD4^+$ lymphocytes are still predominant but the number of $CD8^+$ cells, which are located at the periphery of the granuloma, increases slowly. These $CD8^+$ cells progressively spread throughout the granuloma. Interestingly, a number of B cells can also be detected in the granulomas.

Human susceptibility to mycobacterial infections

In general, every case of tuberculosis has a particular degree of severity. This is basically determined by the virulence levels of each *M. tuberculosis* strain, but several factors may also predispose individuals to acquire and suffer from a more severe mycobacterial disease.

Tom Ottenhoff (Leiden University Medical Centre, Leiden, The Netherlands) described human genetic deficiencies that cause the inability to produce or to respond to INF-γ. As these alterations block cytokine circuits that are essential for the efficient control of the disease, patients were hypersensitive to mycobacterial and salmonellae infections (de Jong *et al*., 1998). These deficiencies affect type-1 cytokine receptors (Ottenhoff *et al*., 2000). In normal conditions, infected macrophages produce IL-12 and IL-18 that stimulate INF-γ production by T-helper and NK cells. This in turn activates microbicidal mechanisms in the macrophage, which control and eliminate the intracellular pathogen. Complete INF-γR1 or INF-γR2 deficiencies owing to null mutations or deletions in the 5′ part of the gene cause severe infections following infection with mycobacteria or salmonella species of low pathogenicity. In these patients, levels of INF-γ and IL-12 are generally low. In contrast, patients with partial INF-γR1 deficiencies (caused for example by a four nucleotide deletion affecting the cytoplasmic domain and the turnover of the receptor) have normal levels of IL-12 but do not respond to INF-γ. Patients with IL-12Rβ deficiencies may have less severe infections, caused for example by the vaccine strain *M. bovis* BCG (Verhagen *et al*., 2000). These patients have a 10-fold reduction in the levels of INF-γ and are less responsive to IL-12. An IL-12Rβ1-independent pathway (involving MAP kinases) to explain the residual INF-γ levels has been proposed.

As well as genetic deficiencies, other factors that affect the fitness of the immune system must be considered.

Immunocompromized patients generally have more difficulty controlling any infectious disease efficiently, particularly mycobacterial infections, because they target cells of the immune system for prolonged periods of time. Acquired immune deficiency syndrome (AIDS) patients are particularly prone to mycobacterial infection, especially by *M. avium* or M*. tuberculosis*. The high frequency of M. tuberculosis–HIV co-infection has complicated the tuberculosis pandemia enormously. Julie Davies Turner (Centers for Disease Control and Prevention, Atlanta, GA, USA) presented preliminary results suggesting that there is a synergy between both pathogens, which means that the replication of either pathogen induces the amplification of the other. Phosphorylation of certain signalling proteins is the common feature that leads to the synergy of these pathogens.

New vaccines for an old disease

Owing to the variable protective efficiencies of the different subsets of the BCG vaccine, a new generation of vaccines against tuberculosis is urgently needed. Several approaches can be used to develop new vaccines; one of which is the use of subunit vaccines. The ESAT-6 antigen belongs to a family of low-molecular-weight secreted proteins present in *M. tuberculosis* culture filtrates that retain the ability to trigger the production of IFN-γ in mice and cattle (Brandt *et al*., 2000). When the ESAT-6 antigen is administrated with an optimized combination of adjuvants, it induces a strong immune response capable of controlling infections equally as well as the BCG vaccination. Antigen 85B is another immunodominant antigen (Belisle *et al*., 1997) that is a promising vaccine candidate because it has been proven to confer protective immunity in animal models. Peter Andersen (Statens Serum Institut, Copenhagen, Denmark) demonstrated that a subunit vaccine constructed by fusing antigen 85B to the ESAT-6 protein induces a strong immune response in mice, conferring high levels of protection similar to those conferred by BCG vaccine (Olsen *et al*., 2000). This recombinant protein conferred immunological memory that remained stable for at least 30 weeks.

It has recently been shown that non-peptide antigens play an important role in the antimycobacterial immune response. For example, Marc Boneville showed that the CD1 antigen-presenting pathway mediates glycolipid presentation to T cells, and that the γδ-T cells are activated by phosphorylated compounds. Also, glycolipids are associated with cording and virulence. Furthermore, in many lipoproteins, the antigenic properties are mainly as a result of the associated lipids. These and other findings have created the possibility of exploring non-peptide antigens as new vaccine candidates because they may confer protective immunity against tuberculosis.

Considerable progress has been made in the development of subunit and non-peptide vaccines, some of which have generated very promising results in experimental models. However, the goal is still to obtain a vaccine that is better than the currently used live *M. bovis* BCG vaccination. An alternative approach involves the generation of new live attenuated vaccine candidates or new recombinant BCG strains. Thus, the development of genetic tools and information on virulence factors, persistence and immunogenicity could lead to the construction of genetically stable and well-defined strains with better immunogenic properties than BCG.

Conclusions

The presentations in this Workshop demonstrated that we have made steady progress in understanding the biology of tuberculosis from a molecular point of view and that we are now ready to study tuberculosis in humans in addition to animal and *in vitro* models. Field studies will be undertaken with new tools derived from molecular studies.

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