Instituto Juan March
de Estudios e Investigaciones

117 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Approaches to Tuberculosis

Organized by

B. Gicquel and C. Martín

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P. Andersen
C. E. Barry III
E. Boettger
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Instituto Juan March (Madrid)

INDEX

Instituto Juan March (Madrid)

Instituto Juan March (Madrid)

PAGE

Instituto Juan March (Madrid)

Introduction

B. Gicquel and C. Martín

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More than one hundred years after the discovery of *Mycobacterium tuberculosis* as the etiological agent of tuberculosis by Robert Koch, tuberculosis is still today one of the leading causes of death worldwide associated in developing countries to AIDS and with alarming increasing levels of drug-resistance.

Differently to other human pathogens, *M. tuberculosis* use a slow growing strategy. A third of the world's population has latent infection. The resurgence of concern about tuberculosis has resulted in a better understanding of parasite-host interactions.

Recent advances in the molecular biology and genetics of mycobacteria have resulted in the development of genetic tools for the manipulation of tubercle bacilli and provided the complete sequence of its genome. Cell biology contributes today to the understanding of the entry of bacteria through specific receptors, survival, phagosome trafficking and activation of signal transduction pathways.

The participants in this remarkably exciting workshop represented an international group of prominent investigators in tuberculosis with diverse interests, backgrounds, research strategies and viewpoints. The workshop has succeeded in updating several topics in tuberculosis fields of genetics and genomics; mycobacterial resistance; *M. tuberculosis* as an intracellular pathogen; tuberculosis immunity and new vaccines and futures perspectives.

The Juan March Institute has provided an excellent forum for the discussion of the recent molecular advances in tuberculosis research that could contribute to the future control of tuberculosis in XXI century.

B. Gicquel and C. Martín

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Tuberculosis as a global problem in the 21st century

Douglas B Young

Imperial College, London, UK

At the start of the 19th century, tuberculosis claimed the lives of around 500 per 100,000 of the population of England and Wales every year. By the end of the century tuberculosis mortality had declined 5-fold; presumably in response to favourable changes in lifestyle. During the same period average life expectancy increased from around 35 to 45 years. The declining trend continued through most of the 20th century, accelerated by the introduction of effective treatment. In contrast, despite socioeconomic improvements in developing countries in the second half of the 20th century (average life expectancy rose from 40 to 60 years), there was no corresponding decline in tuberculosis, with the HIV epidemic in fact triggering a dramatic increase over the last two decades. What are the factors that contributed to the decline in 19th century Europe but were missing from the developing world in the 20th century? In considering Molecular Approaches to Tuberculosis in the 21st century, it is useful to reflect on successes and failures in the last two centuries. In particular, it is important to appreciate the dominant influence of sociological factors in tuberculosis and to try and understand these in terms of the cellular and molecular models we use in the laboratory.

Development of drugs and vaccines for control of tuberculosis is severely disadvantaged by the current structure of the pharmaceutical industry. At best, tuberculosis is likely to provide a market with marginal profits, and any active involvement on the part of industry will depend on imaginative public-private funding arrangements. In considering how the pharmaceutical industry may develop in the 21st century, it is interesting to envisage the possibility of transition from a system in which profits depend on the willingness of sick people paying to get better, to a system in which healthy people pay to remain healthy. Insights from human genome analysis could provide the science base to underpin such a transition to preventive healthcare. This environment would favour development of a new generation of 'preventive therapy' for tuberculosis. In combination with reliable tests for infection and realistic risk estimates for disease development, there could be a market for novel drugs or vaccines capable of preventing disease in infected individuals. Such an intervention would be useful for control of disease import into low incidence countries as well as contributing to population-based control programmes in high incidence countries. Post-infection, pre-disease will be an important area for future research.

It is unlikely that any single intervention will on its own allow global control of tuberculosis. Treatment-based intervention has proven highly effective within the context of a declining epidemic, but it seems likely that it will be the synergy of environmental factors together with drugs and vaccines that holds the key to success. In this context, there is a notable lack of synergy between current drug and vaccine-based interventions. Drugs such as isoniazid are optimally effective against actively-growing mycobacteria, and their action tends to be diminished rather than enhanced by immune-mediated control. In searching for new tools for tuberculosis control, it will be useful to screen for interventions which can be combined in order to generate enhanced efficacy.

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Session 1: Genetics and genomics
Chair: Brigitte Gicquel

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Comparative mycobacterial genomics

S.T. Cole

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Mycobacterium tuberculosis, the scourge of humanity, is one of the most successful and scientifically challenging pathogens of all time. The complete 4.41 Mb genome sequence is available for H37Rv, the paradigm strain for the slow-growing *M. tuberculosis* complex (1). Bioinformatic analysis led to the identification of ~4,000 genes in the genome sequence, and provided fresh insight into the biochemistry, physiology, genetics and immunology of this much-feared bacterium. The information and knowledge thus obtained is now catalyzing the conception of new prophylactic and therapeutic interventions against tuberculosis, and enhancing our understanding of the biology of the tubercle bacilli. The abundance of genes predicted to be involved in lipid and polyketide metabolism suggests that lipolysis is likely to be the major metabolic source of energy and carbon. Prominent amongst the "orphan" genes were two large families encoding novel, glycine-rich proteins of repetitive structure, the PE and PPE proteins. Over 8% of the genome is devoted to the production of these curious proteins of unknown function and this implies that they must play an important biological role.

Comparative genomics of other members of the *M. tuberculosis* complex, using BAC-arrays, has uncovered a series of variable loci that may be responsible for a variety of phenotypic differences including host range and virulence (2). In parallel, the 3.2 Mb genome sequence of the related leprosy bacillus, *Mycobacterium leprae*, has been determined (3). Comparisons of the two sequences are mutually enriching and have revealed numerous pseudogenes and extensive genetic decay in *M. leprae*. Genome downsizing may account for the exceptionally slow growth of this unculturable pathogen while the evolution of *M. leprae* has naturally defined the minimal gene-set for mycobacteria.

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Virulence genes of *M. tuberculosis*

Christophe Guilhot

Although *Mycobacterium tuberculosis* causes more deaths worldwide than any other single pathogen, the mycobacterial factors important for its virulence are largely unknown. Increasing our knowlegde of these virulence factors and their mechanisms of action is essential if we are to understand mycobacterial pathogenesis. We used several complementary approaches to identify virulence genes. We first used of two different reporters to identify *M. tuberculosis* genes induced during macrophage infection, or encoding exported proteins that might come into contact with the host cell (1, 2). These genetic strategies enabled us to identify of a set of genes that may be important in virulence. However, mutagenesis experiments are required that these genes are important *M. tuberculosis* pathogenicity. One of these genes, *erp*, was studied in more detail and shown to be essential for *M. tuberculosis* virulence (3). We also used a more direct approach, the Signature-tagged Transposon Mutagenesis method, in *M. tuberculosis* (4). A library of signature-tagged mutants of this bacterium was constructed and screened for mutants affected in multiplication within the lungs of mice during the first three weeks of infection. Sixteen of 1927 mutants tested had attenuated virulence. The insertions harboured by the selected mutants were mapped in the *M. tuberculosis* genome and most of the mutated loci appeared to be involved in lipid metabolism or transport across the membrane. Four independent mutations were identified in a 50 kb region of the chromosome. This region contains polyketide synthase genes involved in the biosynthesis of phtiocerol dimycoserosate (DIM) (4). This lipid has been shown to be located in the outer layer of the mycobacterial cell envelope (5). Two systems, an ABC transporter and a transporter of the Resistance-Nodulation-Cell Division superfamily, are important for the proper distribution of DIM. Mutations in genes encoding these two transport systems led to accumulation of DIM in the cytoplasm and plasma membrane subcellular fraction. In addition to being attenuated in mice, mutants affected in the production or distribution of DIM were more permeable to hydrophobic compounds and more sensitive to detergent. These experiments demonstrate for the first time that extractable lipids are involved in the permeability barrier and virulence of *M. tuberculosis*.

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Deconvoluting the biosynthesis of siderophores from *Mycobacterium tuberculosis*

Luis E. N. Quadri

Mycobactins are siderophores produced by *Mycobacterium tuberculosis* and other pathogenic and saprophytic mycobacteria. Siderophores are low molecular weight iron-binding compounds utilize by microorganisms to acquire iron, an essential nutrient for bacterial growth. Mycobactins have been shown to "steal" iron from human iron-binding proteins and to be essential for multiplication of *Mycobacterium tuberculosis* inside of macrophages, the primary site for residency and growth in the infected human host. These observations validate the relevance of these siderophores for *Mycobacterium tuberculosis*. The backbone of mycobactins is a peptide-polyketide hybrid structure assembled via a multiple-carrier thiotemplate mechanism. Several peptide synthetases and polyketide synthases are believed to be involved in the assembly of the backbone of these complex siderophores. The objective of the studies addressed in this presentation is to deconvolute the enzymatic pathway that leads to the biosynthesis of mycobactins produced by *Mycobacterium tuberculosis* and other mycobacterial pathogens. The progress in the elucidation of the different steps in mycobactin backbone biosynthesis that result in the assembly of the first third of the peptide-polyketide hybrid molecule will be presented. Insights into the biosynthesis of the virulence-conferring mycobactins will facilitate the rational development of new therapeutic strategies that could be useful in combating *Mycobacterium tuberculosis* infections by inhibiting iron acquisition.

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Session 2: *M. tuberculosis* as an intracellular pathogen
Chair: Douglas B. Young

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Non-conventional lymphoid immunity against mycobacteria

Marc Bonneville

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Cellular immunity against infectious agents involves several T lymphocyte subsets that differ by their phenotype, their mode of antigen recognition and their functional features. Besides «mainstream» $CD8^+$ and $CD4^+$ $\alpha\beta$ T cells, that recognize peptidic antigens bound to highly polymorphic major histocompatibility complex (MHC) molecules, several recent studies have identified in humans and rodents at least two other T cell subsets reacting against an heterogeneous set of nonpeptidic compounds. One subset, which expresses $\gamma\delta$ T cell receptors (TCR) found predominantly on peripheral blood lymphocytes of human adults, recognizes in a yet ill defined fashion a set of phosphorylated compounds broadly expressed by bacteria and parasites. The other subset, which in most cases expresses $\alpha\beta$ TCR, recognizes diverse hydrophobic antigens of mycobacterial origin, that are presented by a family of monomorphic molecules called CD1. Here will be reviewed our current knowledge about the structure of mycobacterial antigens recognized by $\gamma\delta$ and CD1-restricted T cells, with a particular focus on the latest insights about the molecular basis of non-peptidic antigen recognition by these non-conventional lymphocytes.

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Adaptive responses in the *Mycobacterium*/macrophage interplay

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Cornell University

Pathogenic species of *Mycobacterium*, including those of reduced virulence such as *Mycobacterium avium*, exhibit the capacity to survive in macrophages both in culture and within the infection foci in the host. This capacity is dependent on the microbe's ability to respond to, and regulate, its immediate environment both at the level of the host cell as well as the surrounding tissue.

The vacuole in which viable *Mycobacterium* survive shows limited acidification (pH 6.2), does not fuse with lysosomes, and yet remains highly dynamic, stabilized within the rapid recycling pathway of the macrophage (1,2). Early vacuoles show MHC class II molecules, however, these are peptide-loaded and are, at least in part, surface-derived. In more mature vacuoles, greater than 24 hr old, the vacuolar membrane is devoid of MHC class II molecules indicating that the vacuole lies on the efferent arm of the recycling pathway. Activation of the macrophage by cytokines such as IFN-γ results in a series of alterations in the physiology of this compartment. The vacuoles now acidify close to pH 5.2 (3), they show processing of lysosomal hydrolases, and they acquire both class II MHC molecules and H2-M molecules indicating that they now intersect with the trafficking pathway taken by "presentation-competent" MHC class II molecules (4). Moreover, the alterations lead to bacterial stasis and, for some mycobacterial species such as *M. avium*, to bacterial death.

Obviously pathogenic mycobacteria have a vested interest in modifying this response. This is achieved at at least two levels. First the bacilli actually suppress the infected macrophage's ability to respond to activating cytokines (5), and second the granuloma itself is established in such a manner as to limit lymphocytes capable of delivery of activating cytokines to the margin of the granuloma. Such behavior appears to be enhanced,if not mediated directly, by the mycobacterial cell wall lipids that are released into the host cell. The release of such copious amounts of lipid must be very "expensive" to the bacterium in terms of energy, therefore for such a phenomenon to be retained though evolution the "payback" must indeed be great. *Mycobacterium* spp. release at least 7different cell wall lipids into their host cell. These lipids intercalate into the host cell membrane, traffic through the cell, an aggregate into multi-vesicular compartments (6). The multivesicular compartments resemble MIIC compartments and are capable of disgorging their contents into the external milieu. The exocytosed vesicles are internalized by bystander macrophages, that are induced to spread the non-responsive phenotype enabling the bacterium to spread its influence beyond the confines of the host cell. These lipids, in isolation, can induce granuloma-like structures on introduction into mice.

Finally, many labs are interested in identification of gene products that are expressed preferentially inside the host macrophage. However, as many of these genes represent metabolic adaptations to alternate nutrient sources it has been difficult to identify the significance of these data. In collaboration with John McKinney, Bill Jacobs, and Jim Sacchettini, we have found that the ability of *M. tuberculosis* to mobilize the glyoxylate shunt pathway of lipid catabolism is required for the bacterium to maintain a persistent infection

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(7,8). Mutants that are defective in isocitrate lyase (Δicl) establish infection indistinguishably from wild type bacilli (wt), yet show a slow decline in numbers during the chronic phase of disease. Expression of icl in macrophages in culture correlates directly to the activation status of the cell, and the Δicl mutant is impaired severely for survival in activated, but not resting, macrophages. The data demonstrate that icl is key to maintenance of a persistent infection which is modulated at the level of the host cell. This type of adaptive response to the changing environment experience during the course of the infection stresses the plasticity of the interaction and demonstrates that the bacterium must be capable of sensing, and responding to, alterations in its immediate environment.

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Nonopsonic phagocytosis of *Mycobacterium kansasii* by human neutrophils depends on cholesterol and is mediated by CR3 associated with GPI-anchored proteins

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Receptors involved in the phagocytosis of microorganisms under nonopsonic conditions have been little studied in neutrophils. Complement receptor type 3 (CR3) is a pattern recognition receptor able to internalize zymosan and C3bi-coated particles. We report that antibodies directed against CR3 strongly inhibited nonopsonic phagocytosis of *Mycobacterium kansasii* in human neutrophils. In these cells, CR3 has been found associated with several glycosylphosphatidylinositol (GPI) anchored-proteins localized in cholesterol-rich microdomains (rafts) of the plasma membrane. Cholesterol sequestration by nystatin, filipin or ?-cyclodextrin as well as treatment of neutrophils with phosphatidylinositol phospholipase C to remove GPI-anchored proteins from the cell surface markedly inhibited phagocytosis of *M. kansasii*, without affecting phagocytosis of zymosan or serum-opsonized *M. kansasii*. Antibodies directed against several GPI-anchored proteins inhibited phagocytosis of *M. kansasii* but not of zymosan. N-acetyl-D-glucosamine known to disrupt interactions between CR3 and GPI-proteins also strongly diminished phagocytosis of these mycobacteria. In conclusion, phagocytosis of *M. kansasii* involved CR3, GPI-anchored receptors and cholesterol. In contrast, phagocytosis of zymosan or opsonized particles involved CR3 but not cholesterol or GPI proteins. We propose that CR3, when associated to a GPI-protein, relocates in cholesterol-rich domains where *M. kansasii* are internalized. When CR3 is not associated with a GPI-protein, it remains outside of these domains and mediates phagocytosis of Z and opsonized particles but not of *M. kansasii*.

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Macrophage phagocytosis: from genes to function

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The immune system of vertebrates consists of innate and adaptive components that differ in their mechanisms of pathogen recognition and the immediacy of their responses. The innate immune system is the first line of defense. It uses microbe line-encoded pattern-recognition receptors that recognize the conserved molecular structures expressed in pathogens but not in the host. The adaptive immune system has exquisite specificity mediated by antigen receptors that are generated by gene rearrangement. However, the development of an effective adaptive response takes time, and it is governed by elements of the innate immune system.

Macrophages represent the cornerstone of the innate immune system. They comprise 15% to 20% of the cells in most organs and are particularly abundant at the sites of pathogen entry, such as lung, skin, gut, and genitourinary tract. On recognition of a pathogen, the macrophage engulfs it and then undergoes a program of activation: this results in a differentiated cell that is capable of killing the microbe and initiating the adaptive immune response through antigen presentation and cytokine production. The activated macrophage is also the archetypal inflammatory cell. On the one hand, it produces a wide spectrum of inflammatory mediators that establish the precondition for host defense and healing. On the other hand, when macrophages are activated in an uncontrolled manner, they become the effector cells in a wide variety of inflammatory conditions, including toxic shock. Bacterial lipopolysacchharide (LPS), a major cell-wall component of Gram-negative bacteria, is the prototypic macrophage-activating factor.

The coupling mechanisms between macrophage phagocytosis and the induction of the inflammatory response will be the focus of my presentation. I will also discuss a multidisciplinary approach utilizing dominant negative mutations and gene deletion strategies. In addition, I will present data on the characterization of a battery of antiphagosomal monoclonal antibodies, an approach directed toward the discovery of novel elements that regulate phagocytosis. Finally, I will describe the role of the Toll-like receptors in defining the nature of the threat within the phagosome.

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Interactions between *Mycobacterium tuberculosis* and host macrophages and dendritic cells

M. J. Colston

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Macrophages and dendritic cells play key roles in initiating immune responses against *M.tuberculosis*. Macrophages phagocytose the organism at the site of entry. Following phagocytosis, the mycobacterium is able to survive within the macrophage. However, this initial interaction between the mycobacterium and the macrophage involves a complex "cross-talk" in which gene expression of both cells is altered. Using a combined proteomic and transcriptomic approach we have shown that, immediately following infection with *M.tuberculosis*, there is a wide-scale switching on of macrophage genes encoding proteins involved in cell migration and homing. Thus, one of the key roles of the macrophage immediately following infection is to attract cells involved in the innate and acquired immune response to the site of infection.

Dendritic cells play a key role in priming anti-mycobacterial T-cell responses. In peripheral tissues, dendritic cells are present in an immature state, and are poor stimulators of naïve T cells. However, they are specialized in antigen uptake, and after interaction with pathogens or inflammatory products they are induced to migrate to the paracortical T cell areas of draining lymph nodes for priming specific T cells. Thus the activation of dendritic cells by mycobacterial antigens, either by direct infection with *M.tuberculosis* or by acquisition of antigen from infected macrophages, is likely to play a key role in generating an effective acquired immune response. We, and others, have shown that dendritic cells can be efficiently infected with *M.tuberculosis*. We have also shown that infected dendritic cells that have been irradiated are capable of adoptively transferring specific immune responses and protective immunity against experimental infection with *M.tuberculosis* (1). We have now gone on to investigate the requirements for successful transfer of protective immunity by antigen-primed dendritic cells and we are investigating the activation of dendritic cells by *M.tuberculosis* and its product and the acquisition of mycobacterial antigens from infected macrophages. We are also investigating the possibility of manipulating dendritic cells activation states *in vivo*.

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Session 3: Tuberculosis drug resistance
Chair: Stewart T. Cole

Instituto Juan March (Madrid)

The mycobacterial cell wall

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Our understanding of the mycobacterial cell wall has developed rapidly over the past few years from primary structural elucidation to definition of biochemical pathways and, more recently, to an understanding of the genetic basis of biosynthesis and turnover. Only now is some comprehension of the broad physiological role of the mycobacterial cell wall emerging, especially in the context of permeability and contribution to host-pathogen interaction. There are two major elements to the cell wall structure, a core common to all mycobacteria and some closely related genera, and a variable component composed of free lipids, carbohydrates, and proteins. The order in which these are arranged is a matter of much debate, not helped at all by innumerable and contrasting models. The core consists of peptidoglycan not unlike that of *E. coli* in quantity and structure, except that the majority of muramic acid derivatives are N-glycosylated. Attached to the 6 position of occasional N-glycolylmuramic acids are cell wall "linker units", not unlike those in gram-positive bacteria, but structurally distinct in structure, P-N-Ac-GlcNAc-Rha. Attached to the Rha of the linker unit is a linear galactan consisting of about 30 Gal*f* units in alternating 1→5 and 1→6 linkage. Attached in turn to the galactan are one or more chains of the highly branched arabinans, and the terminal Ara*f* units of the arabinan have the esterified mycolic acids attached. It has been proposed that the mycolic acid residues are oriented perpendicular to the cytoplasmic membrane and are complemented by free lipids such as in the case of *Mycobacterium tuberculosis*, the trehalose mycolates, the phthiocerol-containing lipids, and some phospholipids, to form a distinct lipid barrier, the basis of the relative impermeability of mycobacteria to various solutes. However, more and more evidence is emerging that this unique lipid environment can be transversed by porins, providing access for hydrophilic nutrients. While our knowledge of whole wall assembly is in its infancy, we now know much about the biosynthetic origins of the individual entities. In particular, *de novo* synthesis of mycolic acids and the unique interplay of two fatty acid synthetases are well understood. We know that the linker unit, followed by assembly of galactan and some of the arabinan, all take place on a polyprenyl-P carrier allowing exodus from cytoplasm to cell envelope. We know of the biochemical and genetic basis of the Gal*f* and Ara*f* units. And all of this knowledge provides the underpinning for concerted efforts by many laboratories at new anti-TB drugs discovered addressing these extraordinary biochemical and permeability features of mycobacterial cell walls.

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Molecular basis of resistance

E.Boettger

During the past years significant knowledge concerning the mechanisms of drug resistance in mycobacteria has been gained. So far genes encoding drug-modifying enzymes, located on plasmids or transposons, have not been found in *M. tuberculosis*. Resistance to a variety of drugs was found to be exclusively due to chromosomal mutations (e.g., 1-4). Moreover, multidrug-resistant strains have been shown to arise by sequential acquisition of such mutations.

A number of observations complicate the understanding of mycobacterial drug resistance.

1. Standardized short-course chemotherapy (SCC) regimens with first-line agents were found to be adequate for some patients with drug resistant tuberculosis, including multidrug resistance (5). It is difficult to grasp the successful treatment of multidrug-resistant tuberculosis using SCC, which as determined by outcome was an adequate treatment for 50-60 % of the multidrug-resistant cases. How to explain the heterogeneity in treatment outcome of multidrug-resistant tuberculosis involving at least resistance to the two most potent drugs rifampicin and isoniazid? How to reconcile successful chemotherapy with apparent drug resistance?

2. The policy endorsed by the WHO (World Health Organization) to base the containment of multidrug-resistant tuberculosis on a strategy of preventing the generation of new multidrug-resistant tuberculosis cases has been shown not to control multidrug-resistant tuberculosis in countries in which multidrug-resistant tuberculosis is already present (5, 6). The failure to control already present multidrug-resistant tuberculosis by preventing the generation of new multidrug-resistant cases using implementation of the DOTS strategy implies that multidrug-resistant tuberculosis in persons with newly diagnosed tuberculosis must be the result of primary transmission of multidrug-resistant tuberculosis in the community.

Two mechanisms help to understand the biological implications of mycobacterial drug resistance: 1. the predictive value of drug susceptibility testing, 2. the fitness cost of chromosomal drug resistance. The hypothesis can be put forward that the seemingly paradoxical heterogeneity in treatment outcome of multidrug-resistant tuberculosis may be in part due to limitations in the clinical predictive value of in-vitro susceptibility testing on the basis of unique but mistakenly used techniques in diagnostic mycobacteriology (7). The ongoing primary transmission of drug-resistant tuberculosis indicates that the respective mutations conferring resistance do not affect fitness nor relative rates of transmission. In contrast to resistance mediated by exogenous genetic elements chromosomal drug resistance conferring mutations commonly carry no fitness cost (8). These findings do not only explain certain aspects of mycobacterial drug resistance but also have important consequences for the future control of multidrug-resistant tuberculosis.

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Molecular epidemiology of multidrug resistant tuberculosis

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An alarming increase in multidrug-resistant tuberculosis (MDR-TB) has been reported in different countries. In the last years outbreaks of MDR-TB have been reported in hospitals and prisons in the United States related to HIV positive patients. Recently MDR-TB outbreaks with similar characteristics have been reported in Western and Eastern European countries, especially in Prisons (Portaels *et al* 1999). World Health Organisation reports a prevalence greater than 3% of MDR-TB in different geographical settings all around the world, but we know very little about genetic characteristics of these strains (Pablos-Mendez *et al* 1998).

Specific resistance genotypes has been associated with decrease of growing and persistence of *M. tuberculosis* in mice and guinea pigs animal models (Li *et al* 1998). Systematic molecular epidemiological studying human isolates suggest that *M. tuberculosis* resistant genotypes are less transmitted than the full sensitive ones (Van Soolingen *et al* 1999). However outbreaks of MDR stains has been described as is the case of the "W" strain in the USA belonging to Beijing family (Bifani *et al* 1996).

Systematic fingerprinting of MDR-TB strains it is extremely useful for outbreaks detection and surveillance purposes. In Spain all MDR-TB isolates are typed since 1998 (Samper *et al* 2000). Most of MDR TB cases are secondary to treatment and majority of MDR *M. tuberculosis* strains were not further transmitted to other patients but a single MDR-*M. bovis* strain "B" was isolated in 11. In the last 5 years same strain "B" caused outbreaks implicating more than 100 cases mostly HIV positives.

When national and international genotype database are compared, it is possible identify specific MDR-TB genotypes (Samper *et al* 1997). By using molecular epidemiology methods we try to understand if MDR-TB outbreaks are caused by particular strains with unknown selective advantage as in the case of the genotype family strains: "Beijing-W" in the USA "M" in Argentina (Ritacco *et al* 1997) or "B" MDR *M. bovis* in Spain. These findings suggest than particular *M. tuberculosis* complex MDR strains could have, at least, identical virulence than sensitive ones and they are better adapted for MDR- TB transmission.

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Development of a second-generation ethambutol

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We have previously described the synthesis and screening of an initial 100,000-member combinatorial library of diamine analogs of ethambutol. Screening of such large compound collections for appropriate candidates for preclinical and clinical evaluation draws on many different disciplines which must be simultaneously considered and integrated to produce a viable candidate for clinical use. The development of structure-independent tools to drive lead development and optimization depends upon genome sampling techniques such as microarray analysis of transcriptome response to analog treatment and differential protein expression studies for the analysis of proteomic responses to potential therapeutics. These tools facilitate the development of lead series of molecules with interpretable structure-activity relationships and can drive rational drug design programs independent of target-lead cocrystallization. We have used and developed new tools for the analysis of lead molecule effects on bacterial physiology and have used such screens to effectively guide design of second-generation ethambutol like molecules. Diamine analogs with improved potency have been obtained from such screens that operate on the same molecular target as ethambutol and these analogs are potent antibacterial agents *in vitro*. To facilitate high-throughput analysis of lead molecules against infected animals we have also designed and implemented novel *ex vivo* models that more accurately predict *in vivo* efficacy. Second generation ethambutol analogs, such as NIH241, have been shown to have improved efficacy per weight (compared to ethambutol and isoniazid) in *in vivo* aerogenic murine models of tuberculosis when delivered orally. Integrating and designing high-throughput toxicological and pharmacokinetic assays into compound library design and screening will allow efficient identification and advancement of molecules that have properties appropriate for clinical development.

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High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection

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The lungs of cystic fibrosis (CF) patients are chronically infected for years by one or a few lineages of *Pseudomonas aeruginosa*. These bacterial populations adapt to the highly compartmentalized and anatomically deteriorating lung environment of CF patients, as well as to the challenges of the immune defenses and antibiotic therapy. These selective conditions are precisely those that recent theoretical studies predict for the evolution of mechanisms that augment the rate of variation. Determination of spontaneous mutation rates in 128 *P. aeruginosa* isolates from 30 CF patients revealed that 36% of the patients were colonized by a hypermutable (mutator) strain that persisted for years in most patients. Mutator strains were not found in 75 non-CF patients acutely infected with *P. aeruginosa*. This investigation also reveals a link between high mutation rates *in vivo* and the evolution of antibiotic resistance. The similarities between the evolutionary mechanisms of *P. aeruginosa* isolated from CF patients and *M. tuberculosis* will be discussed.

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Session 4: Tuberculosis immunity I
Chair: M. Joseph Colston

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Role of type-1 cytokines and T cells in human mycobacterial infectious diseases

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Studies on patients with idiopathic, severe infections due to poorly pathogenic mycobacteria and Salmonella species have revealed that many of these patients are unable to produce or respond to IFN-γ. This inability results from causative, deleterious genetic mutations in either one of four different genes in the type-1 cytokine cascade, encoding IL-12Rβ1, IL-12p40, IFN-γR1 or IFN-γR2. The immunological and clinical phenotypes resulting from these complete or partial deficiencies in type-1 cytokine (receptor) genes, however, show subtle differences that provide insights into mechanisms of disease susceptibility. A novel pathway of IL-12 dependent, Stat-4 independent IFN-γ production was found in IL-12R deficiency. These findings will be discussed in relation to the observed clinical phenotype of these patients.

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Molecular basis of cytokine circuits in TB

Zahra Toossi

Worldwide tuberculosis (TB) causes more deaths than any other infectious disease, however, the underlying mechanisms of the proficiency of *Mycobacterium tuberculosis* (MTB) as an intracellular microbe, and the pathogenesis of TB are not completely understood. Cytokines produced by mononuclear cells upon exposure to or infection by MTB may be crucial to the pathogenesis of MTB.

Both *in vivo* and *in vitro* evidence indicate that patients with active pulmonary tuberculosis are suppressed in cell-mediated immune responses to MTB antigens, including the production of critical TH-1 cytokines such as IL-2 and IFNγ. On the other hand, the production of these molecules remain strong in the self-contained paucibacillary forms of TB, such as pleural TB. By contrast, the dysregulation of cytokine networks in TB involves the production of excess monocyte cytokines. Several studies report that the stimulated release of the proinflammatory cytokines, TNFα , IL-1, and IL-6 are upregulated during TB. We have found that monocytes from patients with active tuberculosis constitutively express the macrophage deactivating/immunosuppressive molecule, TGFβ, and have an expanded capacity to produce this cytokine upon *in vitro* stimulation by products of MTB. In fact, several components of MTB preferentially induce production of TGFβ. In addition, some mycobacterial components, such as 30kD antigen, bind to host molecules (e.g. fibronectin) and enhance cytokine production *in situ*. On the other hand, the microenvironment of the tuberculous lesions and MTB itself may be instrumental in conversion of TGFβ to its biologically active form. Also, TGFβ induces its own converting enzyme in mononuclear phagocytes. Therefore, overall, it appears that the cytokine milieu of the tuberculous lesion, containing mycobacteria and its constituents, may be particularly biased to excess expression of TGFβ. TGFβ was present in Langhans giant cells and epithelioid cells of tuberculous granulomas of TB patients.

However, the pathogenesis of MTB as an intracellular pathogen is likely to be determined both initially (at the time of MTB infection) and continuously (during TB) by its interface with mononuclear phagocytes and specifically their cytokine profile. Studies which allow the simultaneous investigation of both the host cytokine and MTB responses may be of value in understanding this interaction. We have recently studied changes in the RNA level for MTB 85B and 16S, and expression of TNF α during the first 24 hours of infection of alveolar macrophages and autologous monocytes from healthy subjects using real time quantitative RT-PCR. During the first 24 hours of intracellular infection the intramonocyte 85B mRNA level increased significantly in both cell types concomitant with the induction of TNFα expression. The addition of TNFα increased, and the neutralization of TNFα decreased, MTB 85B expression. In macrophages, but not in monocytes, 85B mRNA expression appeared to be dependent on production of reactive oxygen and nitrogen intermediates. These data indicate that activation of cytokines, such as TNFα, in mononuclear phagocytes subsequent to infection by MTB may in turn amplify mycobacterial metabolism and gene expression.

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Activation of Toll-like receptors by microbial lipoproteins

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Microbial lipoproteins trigger host responses via TLRs. Besides causing disease, mycobacteria have long been recognized for having powerful immunologic adjuvant activity, augmenting both cell-mediated and humoral immune responses. In 1972, a study of the mechanism of mycobacterial adjuvants demonstrated the induction of "soluble mediators", now known to be cytokines, which mediated the augmentation of immune responses (1). One cytokine induced by mycobacteria is IL-12, a powerful signal for the generation of Th1 responses required to eliminate intracellular pathogens, including *M. tuberculosis*. Furthermore, patients with mutations in their IL-12R have increased susceptibility to mycobacterial infection. We therefore investigated the mycobacterial products that induce IL-12 as well as the mechanism responsible for its induction (2). These studies revealed that a lipoprotein, the *M. tuberculosis* 19 kD lipoprotein could activate IL-12 promoter activity and cytokine release.

The 19-kD *M. tuberculosis* lipoprotein is a member of a family of prokaryotic lipoproteins. Lipoproteins have been found extensively in both gram-positive and gram-negative bacteria, as well as cutaneous spirochetes *Treponema pallidum* and *Borrelia burgdorferi*. Profound immunoregulatory functions have been attributed to lipoproteins, including monocyte/macrophage activation. The portion of lipoprotein responsible for its immunologic activity is located in the N-terminal triacylated lipopeptide region. Removal of this lipid element rendered the parent product non-activating, and synthetic lipopeptides could activate B-cells and macrophages. Studies of the *B. burgdorferi* OspA lipoprotein and the 47-kD lipoprotein of *T. pallidum* have demonstrated lipoprotein induction of IL-12 mRNA.

We hypothesized that the 19-kD lipoprotein induced IL-12 via TLRs. The role of TLRs in 19-kD-induced IL-12 production was investigated by cotransfecting the RAW 264.7 macrophage cell line with a TLR-2 dominant negative mutant containing a truncation of 13 amino acids at the C-terminus (3), along with the IL-12 p40 promoter construct. Transfection of varying amounts of the TLR-2 dominant negative mutant inhibited 19-kD lipoprotein-induced IL-12 p40 promoter activation. Similarly, the OspA and the N-terminal lipopeptide of the *T. pallidum* 47-kD antigen activated IL-12 p40 promoter activity by a TLR-dependent mechanism. A mAb specific to human TLR-2 blocked the ability of the19-kD to stimulate IL-12 production from primary human monocytes. Since the deacylated OspA (d-OspA) was unable to activate IL-12 production from THP-1 cells, the fatty acyl moiety, which is genetically and structurally conserved among microbial lipoproteins, appears to be crucial for monocyte activation via TLRs.

To determine whether TLRs are sufficient for activation by lipoproteins, experiments were performed using 293 cells, transfecting the NF-κB responsive ELAM-enhancer. 293 cells do not express TLR-2, nor could they be activated by microbial lipoproteins. In contrast, in stable transfectants of 293 cells expressing TLR-2, microbial lipoproteins induced NF-κB in a

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dose-dependent manner. Together, these data provide evidence that TLRs serve to recognize a diverse family of microbial lipoproteins.

Ability of lipoproteins to stimulate iNOS. To determine whether the TLR signaling pathway stimulated by microbial lipoproteins could be linked to a known macrophage antimicrobial mechanism, we investigated whether the *M. tuberculosis* lipoprotein could activate gene transcription for inducible nitric oxide synthase (iNOS). iNOS is critical for the production of nitric oxide from macrophages, currently the only effective macrophage mycobactericidal mechanism *in vitro* and *in vivo*. Gene disrupted mice revealed that this mechanism was necessary for protection against *M. tuberculosis* in mice. 19 kD and OspA lipoproteins induced iNOS promoter activity in the RAW 264.7 macrophage cell line. Activation was dependent on the fatty acyl moieties, since the d-OspA had no activity. Cotransfection with the TLR-2 dominant negative mutants inhibited the ability of lipoproteins to induce the iNOS promoter, thereby suggesting a role for TLRs in the activation of iNOS by microbial pathogens. Stimulation of monocytes with the 19-kD antigen also induced production of nitric oxide.

Aliprantis et al also demonstrated that microbial lipoproteins induced NF-κB activity via TLR2 (4). Interestingly, microbial lipoproteins induced features of apoptosis in human monocytes, including cell shrinkage and membrane blebbing. Induction of apoptosis was confirmed by TUNEL assay and cell lysis demonstrated according to LDH release. Thus microbial lipoproteins have the ability to induce both TLR-dependent activation of host defense and tissue pathology. This dual signaling pathway is similar to TNFR and CD40 signaling, which can induce both NF-kB activation and apoptosis (5). In this manner, it is possible for the immune system to activate host defense mechanisms, then by apoptosis downregulate the response from causing tissue injury.

The ability of lipoproteins to activate TLRs was further corroborated using the TLR2 gene knockout mouse, which did not respond to microbial lipoproteins. TLR ligand specificity was found to be precise enough to distinguish the natural steroisomer of the lipid moiety of bacterial lipoproteins (6).

The presence of Toll in drosophila indicates that Toll proteins represent a host defense mechanism that has been conserved over hundreds of millions of years of evolution. In mammals, TLRs provide the innate immune system the ability to recognize and react to a wide spectrum of microbial pathogens expressing lipoproteins and lipopolysaccharides. Our data indicate that microbial lipoproteins, by triggering TLRs, can activate innate immune responses including iNOS, a direct microbicidal mechanism. Furthermore, lipoprotein activation of IL-12 leads to activation of an adaptive T cell response required for cell-mediated immunity against intracellular pathogens. It should therefore be possible to develop strategies to activate TLRs in order to induce both innate and adaptive immunity as therapy or prophylaxis for a wide variety of microbial pathogens.

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The temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *Mycobacterium tuberculosis*

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The progression of the immune response in the lung after aerosol infection with *Mycobacterium tuberculosis* is a complex cellular event dominated by macrophages and lymphocytes. Although the phenotype of lymphocytes participating in this response is becoming increasingly well characterized, the dynamic influx of these cells during the infection and their spatial arrangements within the lung tissue are still poorly understood. This study shows that in the first month after aerosol infection with *M. tuberculosis* there was a steady increase in the percentages of total CD3+, CD3+/CD4+ and CD3+/CD8+ cells with consistently higher numbers of CD3+/ CD4+ cells than CD3+/ CD8+ cells. As granuloma formation continued it was found to consist of macrophages, CD4 and CD8 T cells, as well as a smaller number of B cells. Whereas CD4 T cells formed organized aggregates CD8 T cells were fewer and more scattered and tended to more prominent towards the periphery of the granulomas. The possible ramifications of the juxtapositions of these two major T cell subsets is discussed.

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Session 5: Tuberculosis immunity II
Chair: Tom H.M. Ottenhoff

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The host immune response to *M. tuberculosis* infection

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Most (90%) of the individuals infected with *Mycobacterium tuberculosis* do not develop active disease, suggesting that they are able to mount a protective immune response against the mycobacteria. However, in 10% of infected persons, the protective immune response fails to develop or is inadequate, and therefore active, even fulminant disease results. In the infected host, the outcome is determined by the balance between the survival and growth of the organisms and the kinetics and magnitude of the host inflammatory and immune responses that will control the infection. The specific mediators that actually regulate and predict the success of the protective response against mycobacteria have not yet been determined.

To identify the determinants of the host protective response, we have carried out studies in which various clinical isolates of *M. tuberculosis* were used to infect mice by the respiratory route. We observed that some *M. tuberculosis* clinical isolates are more virulent than others in mice, causing early death from the infection. The less virulent clinical isolates induced rapid production of TNF-α and IL-12 both of which are necessary to stimulate maturation of antigen presenting cells and the subsequent generation of a protective Th1 T cell response. In further studies, we have observed that mycobacterial components may affect the induction of these cytokines, thus determining the extent of protection of the host response. For example, we found that the more virulent *M. tuberculosis* clinical isolates contain apolar lipids that induce lower levels of either TNF-α and/or IL-12. In addition, when human monocyte derived macrophages are infected in vitro with recombinant *M. smegmatis* secreting the *M. tuberculosis* 19kDa lipoprotein (*M. smeg* 19kDa), the production of TNF-α and IL-12 is reduced. This observation may explains why in previous studies *M. smeg* 19kDa was not a good vaccine candidate against *M. tuberculosis* challenge. The mechanism(s) by which mycobacterial components can affect cytokine production are now under investigation.

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TB-vaccines and protective immunity

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For a number of years, large resources have been invested in the identification of candidate molecules for inclusion in a novel vaccine against tuberculosis. Various techniques have been exploited and have resulted in the identification of immunologically important antigens such as the immunodominant ESAT-6 antigen and members of the antigen 85 complex.

Today, the availability of the total nucleotide sequence of the *M. tuberculosis* genome enables a post genomic antigen discovery approach based on denotation and screening of complete protein families containing immunodominant molecules. One group of genes sharing properties with ESAT-6 constitute what has been called the ESAT-6 gene family. The genes have from 10 to 35% homology to ESAT-6, approximately the same size, and share genomic organization. The data accumulated so far demonstrate that these molecules are immunodominant antigens strongly recognized in human TB patients as well as in various animal models. Recently, experimental subunit vaccines based on these components in different adjuvants have been demonstrated to promote high levels of protective immunity in animal models.

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Session 6: New vaccines and future perspectives
Chair: Tom H.M. Ottenhoff

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Evasion of host cell defense mechanisms by pathogenic mycobacteria

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Macrophages are professional phagocytes whose function is to clear the circulation from microbial organisms as well as debry resulting from apoptotic events. Following phagocytosis, such material is transferred to the endocytic pathway and eventually transported to lysosomes for degradation.

Pathogenic mycobacteria, including *M. tuberculosis*, are phagocytosed by macrophages but have gained the capacity to circumvent destruction within lysosomes through inhibition of lysosomal delivery. This resistance to lysosomal delivery occurs for living, but not killed mycobacteria and represents a key mechanisms in mycobacterial survival and significantly contributes to the pathology of diseases such as tuberculosis.

In a search for factors involved in blocking lysosomal delivery, we have characterized a host protein, termed TACO (for tryptophan aspartate containing coat protein) that is a crucial factor for mycobacterial survival inside macrophages. TACO is actively recruited to the mycobacterial phagosome by living mycobacteria where it prevents fusion of phagosomes with lysosomes, thereby allowing the mycobacteria to survive.

We are currently analyzing the mechanism of action of TACO, using a variety of cell biological and biochemical approaches. The results of this work might not only contribute to a better understanding of mycobacterial pathogenesis, but also help to elucidate the regulatory mechanisms involved in transport of protein and lipid moieties in eukaryotic cells.

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Gatfield and Pieters, (2000) *Science* 288:1647

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Cellular mechanisms of synergy in HIV/*M. tuberculosis* coinfection

Julie Davis Turner, Manon Deslauriers, Jana O'Kelley, Nicole L. Latorre, and Frederick D. Quinn

Coinfection by *M. tuberculosis* and human immunodeficiency virus (HIV) is a major health problem in the developing world as well as the inner cities of the developed world. The hallmark of this coinfection is synergy between the two infections in which replication of each pathogen induces amplification of the other. We are investigating cellular mechanisms whereby this synergy occurs.

Using a resting peripheral blood mononuclear cell (PBMC) system, we infected cells from normal (PPD-negative, HIV-negative) donors with HIV, *M. tuberculosis* bacilli, or HIV with *M. tuberculosis* bacilli. Infection of the resting cell system with HIV-1/lai or HIV-1/BaL alone yielded only background viral replication (as measured by p24/GAG antigen ELISA, Coulter). In contrast, coinfection of PBMC with HIV and *M. tuberculosis* resulted in 1000-fold increase in viral replication. Flow cytometry of surface antigens showed no induction of activation markers prior to viral amplification, including CD25, CD69, CD71, or HLA-DR. Further analyses of coinfected supernatants for production of cytokines and chemokines (IL-6,IL-8, IL-12, Interferon-gamma, TNF-alpha, TGF-beta, MIP 1-alpha, and RANTES) showed no significant, reproducible differences that could explain the remarkable viral induction.

Subsequent experiments have focused on earlier cell biological events responsible for the activation of viral replication. Using phosphorylation- specific antibodies (BioSource Int.), kinase activity has been analyzed in coinfected cell lysates and compared to singly-infected cultures. Antibodies specific for stress-response, pro-growth, and JAK/STAT pathways are being used in this system. These results will lead to the cellular mechanism for enhanced HIV replication in PBMC during *M. tuberculosis* coinfection. This information will provide additional targets for drug intervention.

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Approaches for new TB vaccines: The European initiative. Perspectives and future research in tuberculosis

Brigitte Gicquel

Tuberculosis (TB) remains an important public health problem worldwide. The incidence is increasing in many countries. The emergence of strains of *Mycobacterium tuberculosis* that are resistant to existing treatments adds a further frightening dimension to the problems of disease control, and highlights the potential for widespread transmission. New drugs that could shorten the treatments, new vaccines more efficacious than BCG, new molecular probes that could help in the surveillance of antibiotic resistance are clearly warranted.

In the context of the fifth framework program of the European Commission (key action "Control of infectious diseases") several research projects are underway, which usually involve collaboration between academic laboratories and industries. The aims are to produce new diagnostic probes, therapeutic treatments and vaccine candidates, that could be evaluated in collaboration with countries suffering from a high TB incidence. This will provide an opportunity to strengthen laboratories working in association with national tuberculosis control programs.

The TB vaccine cluster is one of these projects. It contains 38 groups including two industrial partners. In this case, the specific aim is to identify optimal strategies for generation of vaccine candidates by comparing innovative approaches based on the identification of novel protein antigens, the characterization of non-protein antigens that elicit T cell responses in man, and the construction of live attenuated strains of mycobacteria. New vaccines will be compared alone or in combination in standardized experimental challenge models to identify candidates superior to the existing BCG vaccine. In parallel, the immunological mechanisms underlying vaccine-induced protection will be analyzed in experimental models and in humans. This information will be used to develop immunological tests for initial clinical assessment of new vaccines.

A coordination of the european initiatives with those of other international agencies will accelerate the discovery and the evaluation of new means to fight against tuberculosis.

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Identification of a transmembranar protein encoded by the gene *Pin* of Mycobacteriophage Ms6: role in superinfection exclusion

M. Pimentel, E. Anes, M. Garcia and J. Moniz-Pereira

Studies on the mechanisms of superinfection immunity are important means of revealing bacterial genes that control phage receptors and can help in understanding the process of normal transfer of phage DNA across the plasma membrane. Ms6 is a temperate phage, isolated in our laboratory that infects *Mycobacterium smegmatis* and forms stable lysogens that are immune to superinfection (1). While enable to reveal lytic plaques the derivative transducing phage E1 (2) is able to transduce the kanamycin resistance in other mycobacterial species such BCG, *M.tuberculosis*, and *M.aureum*. A 4,8kb Bgl II restriction segment of Ms6 DNA was sequenced and demonstrated to have a site-specific recombination locus of the phage (3). In addiction M.smegmatis recombinant strains carrying this fragment were unable to be superinfected by Ms6 (4). The inhibition to superinfection to Ms6 was associated with a 978bp SspI-ScaI restriction fragment, which contains an open reading frame that is 486 bp in length and encodes a 162-aminoacid protein. Small deletions into the ORF were made and the resulting recombinant strains became sensitive to Ms6 superinfection. The predicted aminoacid sequence did not reveal any helix-turn-helix DNA binding motif excluding the existence of a repressor but carries a hydrophobic stretch, long enough to span the membrane. To localize this gene product, the ORF coding for the protein was cloned into the *E. coli* expression vector, pTrc His. Fractionation of the cells led to the identification of the recombinant protein in the inner membrane fraction. Certain phage-encoded resistance mechanisms are known to prevent adsorption to the phage receptor, while others are known to act in superinfection exclusion by inhibiting the DNA transfer from the adsorbed phage particle into the cytoplasm. In an attempt to determine the role of this protein we searched for the ability of recombinants to adsorb phage particles, and for DNA uptake. We conclude both that protein Pin is a protein of the inner membrane and is involved in a superinfection exclusion mechanism. As it happens with the Imm protein of bacteriophage T4 the overproduction of the gene product was lethal for Mycobacterium smegmatis.

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A role for $CD4^+CD25^+$ immunoregulatory T- cells on the course of disease in mice infected with *L. major*

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Susceptibility of BALB/c mice to infection with *L. major* has been shown to result from the development of an aberrant TH-2 response that is instructed by an early IL-4 burst from the Vβ4 Vα8 $CD4^+$ T-cells responding to the immunodominant antigen of the parasite, LACK. Little is known about the mechanisms and cell-to-cell interaction involved. We investigated the role of $CD4^+CD25^+$ immunoregulatory T-cells in this model. *In vivo* depletion of $CD4^+CD25^+$ T cells with the anti-CD25 mAB PC61 induced an increased burst of IL-4 mRNA in draining lymph nodes of BALB/c mice at 16 hours after infection and exacerbated the course of disease. Such intervention also led to a more vigorous TH-2 response. A direct demonstration of the role of $CD4^+CD25^+$ T cells was obtained using SCID mice adoptively transferred with splenocytes from BALB/c mice. As already demonstrated, infection with *L. major* five days after reconstitution of SCID mice with 10^7 spleen cells from wild type BALB/c mice led to the development of a polarised TH-1 response and resistance to infection. However, transfer of the same number of cells depleted of the $CD25^+$ subset led to a TH-2 response and susceptibility to infection. This was associated with a more vigorous proliferation of transferred cells *in vivo* in the absence of the regulatory population. Anti-IL-4 treatment abrogated the TH-2 response in these mice. These results demonstrate that the $CD4^+CD25^+$ immunoregulatory T cells modulate the early IL-4 response and the subsequent course of infection with *L. major* in BALB/c mice.

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Cytokine transcripts in pediatric tuberculosis: a study with bronchoalveolar cells

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Pediatric tuberculosis (TB) differs from adult TB in many features. To date, cytokine expression has not been studied in children with TB. The relative amounts of the various cytokines released at the site of infection may be important determinants of tuberculosis (TB) disease development and pathology. We determined cytokine transcripts in bronchoalveolar cells (BAC) recovered from 9 children presenting TB and from 9 children with pulmonary diseases other than TB. An RT-PCR-based method was developed to quantify the mRNAs encoding six cytokines (IFN-g, IL-12, TNF-a, IL-10, IL-4, TGF-b1) known to play key roles in mycobacterial infections. Checking for the presence of mRNA transcripts made it possible to avoid in vitro amplification and provides a direct insight of events at the site of infection. Expression of mRNA encoding TGF-b, TNF-a and IFN-g was statistically significantly higher in BAC from children with TB than in BAC from children with other pulmonary diseases. Whereas the levels of mRNA transcription for TGF-b is high, the levels of mRNA transcription for IFN-g and TNF-a remain low. All children had low levels of mRNA for IL-12(p40).

IL-4 was barely detectable in all cases. Children with miliary TB had high levels of IL-10 transcripts and low levels of mRNA encoding TGF-b. The immunosuppressive cytokines, TGF-b and IL-10, are overproduced in children with non-miliary TB and miliary TB respectively and are probably involved in the progression of the disease. These data suggest that Th1 responses are reduced in children with TB.

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Characterization of an operon from Mycobacterium tuberculosis probably associated to virulence

F. Bigi

There are four highly related operons (mce 1, 2, 3 and 4) in M. tuberculosis genome. These operons contain at least eight ORFs which may code for possible exported proteins, one of them is a putative invasine (Zumarraga, et al., 1999; Gordon, et al., 1999). In this work we study the mce3 operon, present exclusively in M. tuberculosis, suggesting that this locus may play some specific virulence role in human disease. Upstream of mce3 operon, there is a gene that has tipical motifs of regulator genes. To study the role of this gene on mce3 expression, fusions of the mce3 promoter region to b-Gal reporter gene were made, including or not the putative regulator gene. When the regulator gene was not present, the activity of b-galactosidase is increased, suggesting that this gene might down regulate the expression of mc3 operon. The promotor region was mapped by deletions and cloning in a promotor probe vector and by primer extension. Western blot were done in order to know if mc3 operon is expressed in M.tuberculosis and also for determining the subcellular localization of their products. For this purpose, polyclonal rabbit serum were generated to the first ORF expressed in Escherichia coli as fusion proteins with six-histidine tag. These data indicate that this protein localizes in the membrane of the bacilli. At last, mutant strains in first and third ORF of mce3 operon from M.tuberculosis H37Rv were obtained.

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Is the Shwartzman reaction the origin of the intragranulomatous necrosis in tuberculosis?

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One of the well recognized enigmas in the pathogenesis of the human tuberculosis is the origin of the intragranulomatous necrosis. The Shwartzman reaction has been considered as a feasible hypothesis that might explain this phenomenon. Continuous high levels of TNF-a in the tuberculous granulomas have similarities with the "prepared sites" induced by Shwartzman. Again, the sudden increase of *Mycobacterium tuberculosis* in these lesions before the acquisition of specific immunity and thus the local concentration of endotoxins from their cell wall resembles the local inoculation of endotoxins done by the same author. *Interestingly M. tuberculosis* experimentally infected mice with don't reproduce intragranulomatous necrosis. The aim of this study was the induction of this phenomenon following the Shwartman methodology. C57Bl/6 mice infected aerogenically with a virulent strain of *M. tuberculosis* were intranasally inoculated with lipopolysaccharide on day 19 post-infection. Twenty-four hours later, neutrophils infiltrated the lung parenchyma in a significant level, and ten days later necrosis could be detected in the centers of primary granulomas, that showed scanty macrophages and large amounts of collagen on an eosinophilic background. On the other hand, a significant decrease on the concentration of colony forming units could be appreciated 24 hours after LPS inoculation. Afterwards, nonbronchogenic spreading of granulomas increased and higher levels of interferon-gamma mRNA were detected. These results lend support to Shwartzman reaction as the origin of the intragranulomatous necrosis in *M. tuberculosis* infection, and provides a useful tool to improve experimental murine models in tuberculosis.

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Lipid transport systems of *M. tuberculosis*

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The genome sequence of *M. tuberculosis* revealed the presence of a family of 12 predicted membrane proteins designated MmpL (major membrane proteins large). These are transmembrane proteins that belong to the RND family of transporter systems characterized by the presence of 12 transmembrane domains with 2 extracytoplasmic loops located between the first and the second and between the seventh and the eighth transmembrane domains. RND proteins function as efflux pumps in a variety of different microorganisms and are involved in the transport of such substances as antibiotics (MexB of P. aeruginosa), dyes (AcrB of E. coli) or metal ions (CnrA of A. eutrophus). In some cases they function in collaboration with proteins of the MFP family (Membrane Fusion Proteins). These accessory proteins are bound to the cytoplasmic membrane by an N-terminal transmembrane segment or by a lipid anchor. Occasionally there is also an additional OMF protein (Outer Membrane Family) forming a complex that enables drug efflux simultaneously across the cytoplasmic and outer membranes of gram-negative bacteria. *M. tuberculosis* possesses 12 mmpL genes; 11 of them encode for RND-like proteins with 12 transmembrane domains and molecular weights around 100 kDa, while mmpL6 encodes a protein of 42 kDa with 6 transmembrane motifs. These proteins are highly related with identity at the protein level of between 23% for MmpL3 and MmpL7 and 64% between MmpL5 and MmmpL12. *M. tuberculosis* also encodes five MmpS proteins which are 15 kDa with a transmembrane domain near the N-terminus. To elucidate the function of the MmpL and MmpS proteins of *M. tuberculosis*, we have inactivated the genes that encode these proteins by insertion of the hygromycin gene. These mutants were used to determine the role of these proteins in determining resistance to multiple antibiotics, as well as their affect on the transport of mycobacterial cell wall components.

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The role of cytokine mediated "bystander" activation of memory phenotype CD8 T cells in immunity to *M. tuberculosis*

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Immunological memory to *M. tuberculosis* is long lived, but how this is maintained is not understood. In other model systems Tough and Sprent have suggested the importance of cytokine mediated "bystander "activation of memory CD8+T cells (1), but the relevance to this to anti-mycobacterial immunity is unknown. We have established an in vitro culture system to investigate the importance of bystander CD8+T cell activation in response to *M. tuberculosis*. Spleen cells from naïve m-MT mice were cultured in the presence of either anti-CD3, IL-15, intact mycobacteria or their subcellular components. T cell activation was measured by labelling with the DNA precursor bromodeoxyuridine (BrdU) and using 3-colour flow cytometry to analyse co-expression of CD8, CD44 (as a marker of naïve versus memory cells) and BrdU. Addition of either anti-CD3, IL-15 or LPS increased the frequency of BrdU+ CD8 T cells after 48 hours. Under these conditions both CD44 positive and CD44 negative CD8 T cells responded although there was some preference for the CD44+ population. Data will be presented on the effects of BCG, *M.tuberculosis* and their subcellular components on BrdU expression versus other parameters of T cell activation such as intracellular expression of IFN-g. This will be correlated with induction of key cytokines which mediate these events, particularly type I interferons and IL-15 (2).This work is funded by The Action Glaxo Wellcome TB Program.

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Regulation of katG expression upon oxidative stress in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

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Oxidative stress response and protection against reactive oxygen intermediates appear to be implicated in the intracellular survival of pathogenic mycobacteria and their persistence in the macrophage host. Moreover, several elements of oxidative stress response play a role in the innate susceptibility and acquired resistance to the antituberculosis drug isoniazid.

Mycobacteria developed a defensive response to the oxidative stress, which differs from the inducible OxyR-dependent response in Gram-negative bacteria. Consistently, both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* lack a functional *oxyR* gene.

In this work we analyzed the regulation of the *katG* gene, that encodes the catalase-peroxidase, upon oxydative stress in *M. smegmatis*. The region upstream *katG* was cloned and sequenced. Sequence analysis revealed the presence of an *orf*, encoding for a peptide highly homologous to the Fur protein. The *furA-katG* arrangement appears to be conserved among mycobacteria. Transcription of the *furA-katG* region was analyzed by Northern and the 5' of the transcripts mapped by primer extension. Potential promoter regions for both *furA* and *katG* were cloned in a plasmid upstream of the *luxAB* reporter gene and the luciferase activity expressed by the construct was measured. The corresponding regions of *M. tuberculosis* were also cloned. The basal level of transcription, measured in the absence of oxidative stress, was induced upon oxidative stress.

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Control of ribosomal RNA synthesis in mycobacteria growing under different conditions

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The synthesis of ribosomal RNA has been identified as a rate-limiting step in ribosome synthesis and that is therefore having influence on the growth rate regulation. Different number of ribosomal RNA operons (rrn) have been identified within rapidly growing mycobacteria (RGM), thus *M. fortuitum* has two rrn operons per genome similarly to *M. smegmatis*, and *M. chelonae* has a single copy of rrn operon per genome, which makes it similar to *M. tuberculosis* and other slow growers (SGM) at this respect. Differences when comparing rrn operons between mycobacterial species account mainly in the number of promoters controlling their synthesis. This number is ranging from two promoters in *M. tuberculosis* to five promoters in *M. chelonae*. *M. fortuitum* and *M. chelonae* are two opportunistic RGM that share a same total number of rrn promoters, eventhough they differ in the number of rrn operons per genome. We have investigated the differential usage of the various rrn promoters in those two species when growing under different conditions. Three in vitro media have been checked which can be differentiated by their nutrient content: minimal (Kohne-Harris); medium (Sauton) and rich medium (Lemco). Primer-extension analysis was applied on total RNA isolated from mycobacterial cultures. Promoter usage was quantified and results compared with those obtained by quantitative PCR. RNA isolated from mycobacteria recovered from macrophages cultures has been also tested in *M. fortuitum* by using quantitative PCR. Differential usage of promoters has been analysed and discussed.

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Analysis of the humoral immunologic response against four glycolipids from the Mycobacterium tuberculosis cell wall

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The non-peptidic antigens from the Mycobacterium tuberculosis cell wall are the focus of extensive studies in order to find what antigens trigger a immune response against the tuberculous bacilli3,5; and, subsequently, what molecules could be potential protective antigens or serological markers of the infection and/or disease1,4. We have studied the immunological humoral response to four containing-trehalose glycolipids purified from clinical isolates of M. tuberculosis2: diacyltrehaloses, triacyltrehaloses, cord factor, and sulpholipid-I (SL-I). The presence of immunoglobulins G, M, and A were analysed, against these glycolipids, in sera from a wide group of tuberculous patients (92 serum samples, taken before starting the antituberculous treatment) and control people (84 from healthy people inclosed PPD-negatives, -positives, healed and vaccinated; and 52 from non-tuberculosis pneumonia patients) from Spain. The results indicated that there is an elevated significative response in tuberculous patiens with IgG and IgA antibodies compared with the control sera, in all the antigens studied. However, non differences were observed between patients and controls with IgM antibodies. For first time, high specific levels of IgA antibodies are reported in sera from tuberculous patients against these glycolipids, specially to the SL-I antigen (an exclusive antigen from M. tuberculosis). In summary, we conclude that these glycolipids are involved in the immune response against the bacilli; either alone or in combination with other antigenic molecules could be useful serological marker of the disease, or could be perhaps considerate to be inclosed in the design of a new anti-tuberculosis vaccine.

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Development of a *gfp* transposon mutagenesis system in mycobacteria

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Transposition is a powerful tool for identifying mycobacterial virulence genes and studying virulence factors in relation to the host. Our group is working in the construction of modified transposons containing truncated reporter genes that could be used to identify promoters and study their expression. Transposons harbouring promoterless green fluorescent protein (*gfp*) gene will be useful for studying expression of the targeted loci intracellular mycobacteria in both cell cultures and animal models (1,2).

We constructed an IS*1096* derivative (3) transposon harbouring reporter gene *gfp*. Transposon was cloned in TS-sac vector giving pEZ123. This plasmid was tested in *M. smegmatis recA* (HS42) mutant, *M. bovis* BCG and the clinical isolated *M. tuberculosis* MT103. Tn*gfp* transposes randomly in these three strains.

Two Tn*gfp* mutant libraries of HS42 were obtained. Fluorescent mutants were selected at λ=316nm. Sequencing of the insertion point in all mutants showed Tn*gfp* was inserted upstream of rrn gene, encoding 16SrRNA. These strong fluorescent mutants are used as positive controls for fluorescent activated cell sorter (FACS) analysis.

Using FACS we were able to select populations of fluorescent bacteria in *M. bovis* BCG and *M. smegmatis* HS42 from transposon mutant libraries with a single copy of *gfp* gene in the chromosome.

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Signal sequence-independent secretion of the staphylococcal nuclease in *Mycobacterium smegmatis*

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Staphylococcus aureus nuclease has been successfully used as a reporter system for the identification of exported products in Gram positive bacteria such as Lactococcus lactis (Poquet et al., 1998). This methodology has been recently applied to mycobacteria (Downing et al., 1999), but here we provide biochemical evidences that a truncated form of the nuclease is secreted by M. smegmatis independently of a signal sequence. This involves that the nuclease cannot be used as a reporter system to trap signal sequences in mycobacteria, but it opens the way to the characterization of alternative mycobacterial secretion pathways.

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Transcription patron of the *dnaA-oriC* region of *M. tuberculosis* and *M. smegmatis*.

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The regions around *dnaA* gene display the same general organization in many bacteria, and at least six genes, *rnpA-rpmH-dnaA-dnaN-recF-gyrB*, are highly conserved. The regions flanking the *dnaA* gene function as autonomously replicating sequences and acts as the chromosomal replication origin, *oriC*. They contain the DnaA boxes, which have an asymetric 9 pb consensus sequence, 5' c/tTa/gTCCACA. In *Mycobacterium* spp. the *dnaA* gene is preceded by three DnaA-boxes and followed by a further seven, and *oriC* is located in the intercistronic region between *dnaA* and *dnaN* genes. Initiation of chromosome replication in *E. coli* involves the interplay of several molecular interactions at *oriC*. The level of DnaA activity appears to be limiting for initiation and this activity level is apparently established by a combination of the abundance of the protein and its interactions with several negative modulators. There is also transcription in the vicinity of *oriC* that seems to participate in this positive-negative interplay during initiation.

In order to establish the role of *dnaA-dnaN* transcription and the relationship of its transcription to initiation at *oriC*, in this work transcriptional analysis of the *rpmH-dnaA* and *dnaA-dnaN* intergenic regions were determined by primer extension. We identified similar transcriptional start sites of the *dnaN* and *rpmH* genes of *M. tuberculosis* and *M. smegmatis*, but there were differences in the presence and abundance of transcripts in the *rpmH-dnaA-dnaN* intergenic regions. *oriC* of *M. tuberculosis*, but not *oriC* of *M. smegmatis*, showed four main transcripts which are flanking the DnaA boxes. On the other hand, two additional transcripts were identified in the *rpmH-dnaA* region of *M. tuberculosis* with the same polarity as *dnaA* gene transcription. These results suggest that *dnaA* expression and the replication initiation process in the slowly growing member of mycobacteria, *M. smegmatis* are different from those of the rapidly growing members as *M. tuberculosis*.

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Biochemical properties of *Streptomyces* catalase-peroxidase, regulation of its gene by the redox regulator FurS, and implications for the mycobacterial homologues

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Streptomyces reticuli produces a heme-containing homodimeric enzyme (160 kDa), the catalase-peroxidase CpeB (1), which is processed to the enzyme CpeC during prolonged growth. CpeC contains four subunits of 60 kDa which do not include the C-terminal portion of the progenitor subunits. A genetically engineered *cpeB* gene encodes a truncated subunit (lacking 195 of the C-terminal amino acids); four of these subunits assemble to the enzyme CpeD. Heme attaches most strongly in CpeB, least in CpeD. The catalase-peroxidase CpeB and its apo-form (obtained after extraction of heme) catalyze the peroxidation of Mn(II) to Mn(III), independent of the presence or absence of the heme inhibitor KCN. CpeC and CpeD, in contrast, do not exhibit manganese-peroxidase activity. The data show that a bacterial catalase-peroxidase has a heme-independent manganese-peroxidase activity, which depends on the presence of the C-terminal domain (2). The CpeB protein deduced from the cloned gene encodes a protein of 740 amino acids with a molecular mass of 81.3 kDa. It shares most amino acid identities with KatG from *Caulobacter crescentus* and *Mycobacterium tuberculosis*, and PerA from *Bacillus stearothermophilus* (1).

The *cpeB* gene has no promoter of its own. It is co-transcribed together with the adjacent *furS* gene. Physiological and transcriptional studies suggested that FurS (15.1 kDa) acts as repressor in the presence of Mn^{2+} and Fe^{2+} ions. The FurS protein can be converted into one of reduced mobility by treatment with thiol-reducing agents; in the presence of diamide, in contrast, the mobility is enhanced. These properties are due to the presence of redox-responsive cysteine residues. As revealed by gel shift and *in vitro* footprinting studies, only the thiol-reduced FurS protein bound to a motif upstream of the *furS* gene. In the absence of first row divalent ions, the binding site contains 22 bps, in the presence of Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}, it is extended by 18 bps (3). It is interesting that the upstream region of the *furA* gene from several mycobacteria contains a highly similar motif. The predicted mycobacterial FurA regulator shares high amino acid identity with FurS, whose gene is linked to one encoding a catalase-peroxidase (KatG). Thus the data obtained for *S. reticuli* are expected to serve as additional model to elucidate the regulation of mycobacterial catalase-peroxidase genes.

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