MOLECULAR MECHANISMS IN TUBERCULOSIS

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February 19-25, 1995; Tamarron, Colorado

SPONSORED BY ABBOTT LABORATORIES AND THE DIRECTOR'S SPONSORS FUND

Plenary Sessions *Page*

Poster Sessions

Molecular Mechanisms in Tuberculosis

Structure, Biogenesis and Targeting of the Cell Wall of M. tuberculosis

B3-001 THE BIOGENESIS AND GENETICS OF MYCOLIC ACID BIOSYNTHESIS, Gurdyal S. Besra[1], John T. Belisle[1], Kuni Takayama[2], Patrick J. Brennan[1], [1]Colorado State University, Fort Collins, CO 80523, [2]William S. Middleton Memorial Veterans Hospital, Madison, WI 53705.

The mycobacterial cell wall is characterized by the presence of a homologous series of C_{60}-C_{90} α-alkyl, β-hydroxy fatty acids, called mycolic acids, extremely prominent components of the cell wall "barrier," the mycolyl-arabinogalactan complex and the cell surface antigen, trehalose dimycolate. Undoubtedly, the biosynthetic pathways for the biogenesis of this cell wall matrix will involve highly specialized enzymes and enzyme complexes that may be exploited for the development of new anti-mycobacterial drugs. The urgent need to develop new and more effective chemotherapeutic agents to control tuberculosis has lead to our current endeavors in defining the biosynthesis of key cell wall products. Among the most distinctive targets for rational drug design against tuberculosis and atypical mycobacterioses are the cell wall mycolates. The study of their biosynthesis, presumably involves key specialized elongation enzymes; a Δ-5-desaturase; a range of functional group transformation enzymes to introduce different families of mycolates; a Claisen-type condensation reaction; and finally various mycolyl "transporter/exchange" mechanisms. In particular, the roles of our "newly" discovered mycolyl group carrier, the mycolate-containing phospholipid Myc-PL from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* and their implication in the terminal stages of mycolate transfer and relationship to trehalose monomycolate and trehalose dimycolate as possible mycolic acid carriers will be discussed. In addition, the recent characterization of an enzyme purified to homogeneity, from *M. smegmatis* and *M. tuberculosis* H37Ra, by the use of a combination of ammonium sulfate fractionation, anion, cation, and gel-filtration column chromatography, which is also involved in the terminal stages of the mycolate biosynthetic pathway, will also be discussed as a key target for the construction of "knock-out" mutants in *M. tuberculosis*.

B3-002 STRUCTURE AND ROLE OF GLYCOCONJUGATES IN THE MYCOBACTERIAL INTERACTIONS WITH THE IMMUNE CELLS, Germain Puzo, LPTF, CNRS, Dept. Glycoconjugués, Biomembranes, 118 Rte de Narbonne, 31062 Toulouse CEDEX, France.

In tuberculosis, cellular immunity plays a fundamental role in the infection control which is mediated by the immune cell effectors: lymphocytes Tαβ ,Tγδ, and macrophages. Surprisingly, the mycobacteria host cellular targets are the alveolar macrophages. Pathogenic mycobacteria can survive and multiply inside the macrophages despite the various and potent host defenses. Thus macrophages offer to the pathogenic mycobacteria an opportunity to manipulate the immune system to their own advantage. The binding of mycobacteria to macrophages is the first step which leads the pathogen to invade the phagocytes. The mycobacteria route of entry in the macrophages may influence the efficacy with which macrophages can kill mycobacteria. The molecular process of uptake of mycobacteria by macrophages still remains unclear, although several cellular receptors have been implicated. It is established that virulent and attenuated strains of *M. tuberculosis* and the vaccinating strain *M. bovis* BCG bind to human macrophages *via* the CR1 and CR3 complement receptors. Mycobacteria also secrete fibronectin-binding protein including the 30/31 kDa proteins shared by pathogenic and non-pathogenic mycobacteria. Recently, it was proposed that the mannose receptor mediates specifically the binding of the virulent *M. tuberculosis* strains.
The first point addressed in this topic concerns the comparative structural study of the mannosylated lipopolysaccharides: lipoarabinomannan (LAM) and lipomannan (LM), polysaccharides: arabinomannan (AM), mannane from the virulent and avirulent mycobacterial strains. Binding study to the immune cells via biotinylated derivatives by cytofluorimetry wille be presented. The role of these different molecules to mediate the mycobacterial binding to the macrophages will be discussed.
The last part of this topic will be focused on the preliminary characterization of mycobacterial antigens involved in the lymphocyte T activations.

The Proteins of M. tuberculosis

B3-003 SECRETED ANTIGENS AND PROTECTIVE IMMUNITY TO TUBERCULOSIS, Peter Andersen, Statens Seruminstitut, Copenhagen S, Denmark.

Recently a lot of interest has been focused on antigens secreted from live *Mycobacterium tuberculosis* as possible protective antigens. Our attempts to define antigens involved in protective immunity are based upon a defined mixture of secreted antigens contained in a short-term culture filtrate (ST-CF). Experimental vaccines based on ST-CF and a range of different adjuvants have been tested in a mouse model of tuberculosis. The adjuvant Dimethyldeoxydecylammonium chloride (DDA) was chosen for further studies and a vaccine which provides a highly efficient longterm resistance in a mouse model of tuberculosis has been constructed.
To identify protective and promiscuous epitopes, we screen T cell recognition patterns by narrow molecular mass fractions of secreted proteins. In a mouse model of longlived memory immunity this approach has enabled the precise definition of epitopes contained within a 6 kDa secreted antigen recognized by the first wave of immune memory effector T cells. Recent data obtained on this key target and the subset of T cells recognizing this molecule will be presented. The possible relevance of this candidate antigen in a future vaccine against tuberculosis will be discussed.

B3-004 PROTEIN SPLICING AND MYCOBACTERIAL *recA* GENES, Elaine O. Davis, Peter J. Jenner, Harry S.Thangaraj, Steve G. Sedgwick, Patricia C. Brooks, Nichola Thomas and M. Joseph Colston, The National Institute for Medical Research, The Ridgeway, Mill Hill, London, U.K.

Homologous recombination is a widely used approach for investigating gene function. The ability to precisely delete, disrupt or mutate specific mycobacterial genes would provide a powerful tool for investigating the genetic basis of virulence. However, gene disruption by homologous recombination has proved difficult to achieve in slow-growing mycobacteria such as *M.tuberculosis*, although such techniques have proved successful in fast-growing species such as *M.smegmatis.*. Homologous recombination in bacteria is mediated by the RecA protein. RecA is a highly conserved protein which, in addition to its role in homologous recombination, is central to the regulation of more than twenty other genes involved in DNA repair; this co-ordinated expression of genes in response to DNA damage is known as the S.O.S response. Bacteria which lack the RecA protein are highly susceptible to DNA-damaging agents and are recombination deficient.

As a starting point for investigating homologous recombination and the S.O.S. response in mycobacteria we cloned and sequenced the *recA* gene of *M.tuberculosis*. The *recA* gene of this organism is quite different from those of other bacteria, and the RecA protein of *M.tuberculosis* is produced by an unusual protein splicing reaction. Subsequently we showed that the RecA protein of the other major mycobacterial pathogen *M.leprae* is produced by a similar processing event, while the other mycobacteria have normal *recA* genes. The presence of 'protein splicing elements' in the recA genes of the two pathogenic mycobacterial species suggests that protein splicing could play some function for these organisms. Interestingly a second protein splicing element has been identified in an open reading frame of unknown function as part of the *M.leprae* genome project. We are currently developing strategies for investigating DNA repair and homologous recombination in mycobacteria in order to determine whether protein splicing plays any role in gene function in the pathogenic mycobacteria.

B3-005 *M. TUBERCULOSIS* PROTEINS EXPRESSED IN RECOMBINANT MYCOBACTERIAL HOSTS, Douglas B. Young[1], Ying Zhang[1], Jon Cooper[2], Christiane Abou-Zeid[1], and Patrick Brennan[3], [1]Department of Medical Microbiology, St. Mary's Hospital Medical School, London W2 1PG, [2]Department of Crystallography, Birkbeck College, London, and [3]Colorado State University, Fort Collins, CO.

Information generated from genome sequencing projects provides ready access to genes encoding a vast array of mycobacterial proteins. Progress in understanding the role of these proteins in mycobacterial physiology, as possible drug targets, and as antigens, is dependent on the availability of techniques for expression of the functionally active, "native", protein products. We have been investigating the use of two rapid growing mycobacterial hosts - *M. smegmatis* and *M. vaccae* - as convenient laboratory systems for analysing *M. tuberculosis* proteins. Using a shuttle vector system based on hygromycin selection, we have expressed the *M. tuberculosis* superoxide dismutase enzyme at high levels in *M. vaccae*. The enzyme is fully functional and we have been able to use the recombinant product to solve the crystal structure of the protein at 2Å resolution. Site directed mutagenesis has been used to study amino acid residues required for binding of the catalytic iron atom, and those involved in key dimer-dimer interactions in the protein. Expression of *M. tuberculosis* superoxide dismutase in *M. smegmatis* differs from that seen in *M. vaccae*. In this case, the recombinant protein combines with host subunits to form a series of chimeric enzymes. We are also using an *M. vaccae* expression system to study post-translational modification events. The 19kD antigen of *M. tuberculosis* is secreted across the cell membrane and modified by addition of lipid and sugar moieties. By reproducing these events in a rapid-growing mycobacterial host, we have been able to generate sufficient amounts of protein for chemical analysis and are currently investigating use of this model system to allow identification of relevant mycobacterial genes. Use of mycobacterial expression systems for studying protein function and for immune presentation of antigens will be reviewed.

Genetics of M. tuberculosis

B3-006 **MYCOBACTERIAL GENOMICS: PROGRESS AND PROSPECTS,** Stewart Cole[1], Staffan Bergh[1, 2], Karin Eiglmeier,[1] Hafida Fsihi[1], Thierry Garnier[1], Beate Heym[1], Nadine Honoré,[1] William Jacobs, Jr.[3], Lisa Pascopella[3], Wolfgang Philipp[1], Sylvie Poulet[1], Bala Subramanian [3, 1], Institut Pasteur, Paris, France, [2], Royal Institute of Technology, Sweden, [3], Albert Einstein College of Medicine, New York

Mycobacteria have long been the scourge of mankind and the diseases they cause remain a leading source of morbidity and mortality. It is ironic to note that just when multidrug treatment is making a major impact on the incidence of leprosy, tuberculosis is recrudescent in most countries as a consequence of the AIDS pandemic, increased homelessness and declining control programmes. Leprosy results from infection by *Mycobacterium leprae* an obligately intracellular pathogen with a predilection for the peripheral nervous system whereas tuberculosis is caused by *M. tuberculosis*, the first bacterium shown to be the etiologic agent of an infectious disease. Both *M. leprae* and *M. tuberculosis* are slow-growing with doubling times of 13 days and 18 hours, respectively, and *M. leprae* cannot be cultured *in vitro*. The use of genetics to dissect mycobacterial pathogenicity has thus been severely limited and, in consequence, a global approach has been undertaken to tackle this problem.

In the first phase, genomic cosmid libraries, based on both shuttle and conventional vectors, were constructed and clones corresponding to 15 genome-equivalents were ordered into contigs by means of fingerprinting and hybridization mapping. The shuttle cosmid clones are finding application in mycobacteriology and have been useful for identifying genes encoding drug targets, enzymes and various immunogens while the conventional cosmids which carry larger inserts are destined for the second phase of the project, the systematic DNA sequence analysis of the mycobacterial chromosomes. The programme began with *M. leprae* and, at present, its genome is represented by four contigs, accounting for about 2.8 Mb of DNA, on which all of the known genes have been mapped. Gaps between contigs are likely to correspond to unclonable segments. In parallel, similar approaches have been used to construct contigs corresponding to the ~4.2 Mb *M. tuberculosis* genome and this resulted in 12 contigs of ordered clones. In an attempt to obtain map closure these were then correlated with the physical map obtained by pulsed field gel electrophoresis of macro-restriction fragments. The current integrated map consists of a mere six contigs and shows the positions of 57 known genes, 40 loci encoding antigens, 26 PGRS loci and the sites of insertion of IS*6110*, IS*1081* and a novel IS-like element.

Intensive sequence analysis of cosmids bearing DNA from *M. leprae* began in '92 and is being pursued in conjunction with the multiplex sequencing group at Collaborative Research, led by Dr. Doug Smith, and a few academic laboratories. To date the sequences of 34 cosmids, representing nearly half of the genome, have been determined. Analysis of the data has been most informative as over 400 new genes have been identified. These fall into three roughly-equal classes: genes for which precise functions can be assigned, those which show weak similarity to functionally-related genes and others showing no homology. From the sequence data much biological information has been deduced about metabolic pathways, genetic regulons and the nature of potential virulence factors. Curiously, <50% of the *M. leprae* DNA appears to be coding. To facilitate storage and diffusion of genomic data MycDB, a dedicated database, has been established and can be obtained by ftp from ftp.pasteur.fr in the directory pub/MycDB.

B3-007 GENETIC REGULATION OF MYCOBACTERIOPHAGE L5, Graham F. Hatfull, Lisa Barsom, Kelly Brown, Mary Donnelly-Wu, Amy Klann, Janice Lee, Chadd Nesbit, Marissa Pedulla, Carol Peña and Gary Sarkis. Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

Mycobacteriophage L5 is a temperate phage of the mycobacteria and forms stable lysogens in *Mycobacterium smegmatis*. These lysogens contain a single copy of the phage genome integrated into a specific site in the bacterial genome; integration is mediated by the action of the phage-encoded integrase protein in conjunction with a novel host-encoded integration host factor. The state of lysogeny is maintained through the action of a repressor-like protein gp71 which is required for the processes of lysogenic maintenance and superinfection immunity apparently through DNA-binding and transcriptional repression of lytic phage promoters. To monitor the patterns of phage gene expression, we have constructed recombinant derivatives of L5 in which the firefly luciferase gene (FFlux) has been inserted in the late-lytic region close to the left end of the genome. While unexpected, we find that FFlux is not expressed well in lytic growth, but is synthesized during lysogeny when the lytic genes are normally repressed. This paradox has been resolved by the observation that FFlux expression derives from a promoter created at a cloning junction by fortuitous juxtaposition of FFlux and L5 DNA sequences. The 5' end of the mRNA coincides with the first base of the AUG translation initiation codon indicating that *M. smegmatis* can efficiently translate mRNA that does not contain an upstream ribosome binding site. The ability of these L5::FFlux phages to form stable lysogens with high FFlux activity enables the sensitive detection of *M. smegmatis* cells in culture and elucidation of drug susceptibilities. The study of L5 provides insights into the molecular genetics of mycobacteria, tools for the genetic manipulation of the mycobacteria and novel approaches for the diagnosis of tuberculosis and other mycobacterial diseases.

Mechanisms of Drug Resistance in Tuberculosis

B3-008 MECHANISMS OF RESISTANCE TO STREPTOMYCIN IN MYCOBACTERIA, Sheldon Morris and David Rouse, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD

The molecular mechanisms of resistance to streptomycin (SM) in mycobacteria were investigated by studying drug resistant clinical isolates and *in vitro* mutants. Point mutations at codons 43 and 88 of the *rpsL* (ribosomal S12 protein) gene and at positions 491, 512, 513, 516, and 904 of the *rrs* (16S rRNA) gene were shown to be associated with reduced sensitivity to SM. An evaluation of 44 SM resistant *M. tuberculosis* clinical isolates established that 25 of the isolates had *rpsL* mutations, 7 had altered *rrs* genes, and 12 were resistant to SM by yet undefined mechanisms. Streptomycin resistance genetic markers were identified in both singly SM resistant and MDRTB strains suggesting that multiple drug resistance in *M. tuberculosis* primarily results from an accumulation of mutations in individual drug targets. In contrast, no *rpsL* or *rrs* gene alterations have been detected in 20 innately SM resistant *M. avium* strains. Other resistance mechanisms involving permeability barriers or drug export proteins may confer reduced sensitivity to SM in many isolates of *M. avium.*

B3-009 THE NAD PATHWAY OF M. TUBERCULOSIS AS A POSSIBLE TARGET OF ISONIAZID, Maria-Helena Saad[1], Gloria Bonfim[2], and Lee W. Riley[1], [1] Cornell University Medical College, NY, NY 10021, [2]Federal University of Bahia, Salvador, Brazil.

The mechanism of action of isoniazid (INH) against Mycobacterium tuberculosis appears to be multifactorial. The observation that a mutation in the katG gene is associated with high-level INH resistance suggests that the gene product catalase-peroxidase may be important in the antimycobacterial activity of the drug. However, its role in intermediate or low-level INH resistance is not clear. Mutations in the structural or regulatory regions of the gene inhA are associated with resistance to INH and ethionamide, but M. tuberculosis strains that are simultaneously resistant to both of these drugs are uncommon among clinical isolates. A major potential site of action of INH is the NAD metabolic pathway. The Krüger-Thiemer hypothesis proposes that INH is oxidized by catalase into isonicotinic acid (INA), and its amidation product isonicotinamide is incorporated into an NAD analog, which are non-functional as coenzymes. Since M tuberculosis lacks the salvage pathway of NAD biosynthesis, any inhibition of the NAD de novo pathway will effectively shut down the supply of NAD and kill the organism. Our studies suggest that the de novo pathway may indeed be a target of INH. Specifically, we have been studying the enzymes L-apartate oxidase and quinolinate synthetase, which are encoded by the genes nadB and nadA, respectively. Preliminary results suggest that INH may exert its action on one regulatory protein that may serve as an electron-acceptor molecule necessary for the activation of L-aspartate oxidase. Since the catalase is proposed to be involved in the oxidation of INH into INA, and the product of inhA has a potential nucleotide coenzyme binding domain, the hypothesis that the NAD de novo pathway may be a major target of INH is compatible with the other proposed mechanisms of INH action.

Host Resistance Mechanisms

B3-010 T CELLS, MHC MOLECULES AND CYTOKINES IN ANTIMYCOBACTERIAL HOST RESISTANCE, Stefan H.E. Kaufmann, Inge E.A. Flesch and Christoph H. Ladel, Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, D-89070 Ulm, Germany

Protective immunity induced by the antituberculosis vaccine strain, Mycobacterium bovis BCG, is dominated by T cells of TH1 type. These IFN-γ producing CD4 TCRα/β lymphocytes are strongly activated by BCG. In contrast, it is generally assumed that CD8 TCRα/β lymphocytes, which are also of protective value, are insufficiently activated. Similarly, IL-4 producing CD4 TCRα/β lymphocytes of TH2 type remain virtually unstimulated after BCG immunization. The role of TCRγ/δ lymphocytes in antimycobacterial immunity remains enigmatic. We have studied the contribution of cytokines and T cell subsets to the immune response induced by M.bovis BCG in mice.

The activation of TH1 and TH2 CD4 lymphocytes is thought to be regulated by early produced cytokines, in particular IL-10 and IL-12, which promote TH2 or TH1 cell activation, respectively. Surprisingly, BCG vaccination markedly induced production of both IL-10 and IL-12 (although later TH1 cells preponderated). Hence, high IL-10 production alone appears insufficient for driving TH2 cell responses. Although IL-12 synthesis was promptly induced by BCG vaccination, costimulation by IFN-γ and TNF-α was required. Hence, it appears that IL-12 is not the first cytokine produced after M.bovis BCG vaccination.

The course of M.bovis BCG infection was studied in the following gene deletion mutant mice: A$\beta^{-/-}$ mutants (lacking surface expressed MHC class II and hence conventional CD4 TCRα/β lymphocytes), β2m$^{-/-}$ mutants (lacking MHC class I surface expression and hence conventional CD8α/β TCRα/β lymphocytes), TCR-$\beta^{-/-}$ mutants (lacking all TCRα/β lymphocytes), TCR-$\delta^{-/-}$ mutants (lacking all TCRγ/δ lymphocytes), RAG-1$^{-/-}$ mutants (lacking all conventional T and B lymphocytes). M.bovis BCG infection at a dose which was easily controlled by heterozygous controls, was lethal for RAG-1$^{-/-}$, TCR-$\beta^{-/-}$ and A$\beta^{-/-}$ mutant mice. In contrast, the β2m$^{-/-}$ and the TCR-$\delta^{-/-}$ mutants were capable of controlling M.bovis BCG infection, although bacterial growth was slightly exacerbated at later time points. The β2m$^{-/-}$ mice, however, succumbed higher doses of M.bovis BCG infection which were still tolerated by the heterozygous controls. These findings emphasize the central role of MHC class II restricted CD4 TCRα/β lymphocytes in immunity to M.bovis BCG. However, our findings also demonstrate a clear role for MHC class I restricted T cells (probably CD8α/β TCRα/β lymphocytes) in immunity to M.bovis BCG. By inference we assume that M.bovis BCG activates MHC class I restricted CD8 T lymphocytes in immunocompetent mice. Antigen-induced IFN-γ secretion by spleen cells from M.bovis BCG vaccinated mice was markedly affected in both TCR-$\beta^{-/-}$ and TCR-$\delta^{-/-}$ mutants. However, normal levels of IFN-γ could be re-established by admixture of spleen cells from TCR-$\beta^{-/-}$ and TCR-$\delta^{-/-}$ mice. We assume regulatory interactions between TCRα/β cells and TCRγ/δ cells to be essential for efficient IFN-γ production in the BCG system. In conclusion, these findings emphasize contribution of different T cell subsets to the development of optimum protection induced by M.bovis BCG.

B3-011 CYTOKINE REGULATION OF THE CD4 T CELL RESPONSE TO TUBERCULOSIS. Ian M. Orme, Mycobacteria Research Laboratories, Colorado State University, Fort Collins, CO 80523.

Infection of mice with the intracellular pathogen *Mycobacterium tuberculosis* induces the emergence of a Th1-type CD4 T cell response characterized by activated T cells which secrete large amounts of gamma interferon when restimulated in vitro by macrophages presenting mycobacterial culture filtrate proteins. At the early stages of this response, infected macrophages secrete a spectrum of cytokines and chemokines, including the heterodimeric molecule IL-12, which amplifies the CD4 response and promotes formation of the protective granuloma. The sensitized CD4 population then undergoes differentiation into a blast cell population [CD44hiCD45hi]followed by shifts in the CD45 marker expression to a form [CD45lo/neg] that may be representative of the establishment of memory immunity. Using gene disrupted mice, the key protective role of IFN-γ can be demonstrated, in that such mice develop severe, fatal, disseminated disease after exposure to low dose aerosol infections. Similarly, using such genetic manipulation, the roles of other cell populations such as CD8 and $\gamma\delta$ T cells can be investigated. These data will be discussed in the context of the results of vaccine experiments which target both CD4 and CD8 T cell responses in experimental tuberculosis, and the potential for their enhancement by cytokines, or by specific cytokine inhibitors.

Mechanisms of Pathogenesis

B3-012 THE ROLE OF THE MARCKS FAMILY OF PROTEIN KINASE C SUBSTRATES IN PHAGOSOME MATURATION, Alan Aderem, The Rockefeller University, New York, NY 10021.

MARCKS is a widely distributed protein kinase C (PKC) substrate which binds both actin and calmodulin, and which has been implicated in motility, secretion, the regulation of the cell cycle, and transformation. MARCKS localizes to points of actin filament - membrane interaction and appears to regulate actin structure at the membrane, as well as actin membrane interaction. During phagocytosis in macrophages, MARCKS associates with the nascent phagosome together with actin, and remains associated with phagosomes after actin has depolymerized, and phagosome-lysosome fusion has occurred. The role of MARCKS in regulating phagocytosis and phagosome-lysosome fusion will be discussed.

MacMARCKS, a member of the MARCKS family of PKC substrates, is restricted in its expression and is strongly induced in macrophages which have interacted with bacteria. Like MARCKS, MacMARCKS also interacts with calmodulin and actin, and also appears to regulate actin plasticity at the membrane. Upon phagocytosis, MacMARCKS coated vesicles appear to fuse with phagosomes after they have been denuded of actin, and MacMARCKS remains associated with the phagosome till phagosome-lysosome fusion has occurred. The role of MacMARCKS in regulating membrane flow to the phagosome and the effect on virulence factors on this pathway will be discussed.

B3-013 CHARACTERIZATION OF THE MYCOBACTERIUM TUBERCULOSIS PHAGOSOME, Daniel L. Clemens and Marcus A. Horwitz, Division of Infectious Diseases, Department of Medicine, University of California, Los Angeles, CA 90024

M. tuberculosis is an intracellular pathogen that parasitizes human mononuclear phagocytes. Throughout its life cycle in these host cells, M. tuberculosis resides and multiplies in a membrane-bound vacuole or phagosome. The M. tuberculosis phagosome resists fusion with lysosomes and acidification. Beyond that, however, little is known about the phagosome including the composition of the phagosomal membrane or the interaction of the phagosome with other host cell organelles. To learn more about the M. tuberculosis phagosome, we have used the highly quantitative cryosection immunogold technique to examine the composition and maturation of the phagosome, focusing on known markers of the endosomal-lysosomal pathway. For comparison, we have studied phagosomes containing a) wild-type Legionella pneumophila, which like M. tuberculosis, inhibits phagosome-lysosome fusion and phagosome acidification, b) avirulent mutant L. pneumophila, which does not inhibit phagosome-lysosome fusion, and c) polystyrene beads, an inert particle.

The M. tuberculosis phagosome exhibits delayed clearance of MHC class I molecules, relatively intense staining for MHC class II molecules and the endosomal marker transferrin receptor, and relatively weak staining for the lysosome-associated membrane glycoproteins, CD63, LAMP-1, and LAMP-2 and the lysosomal acid protease, cathepsin D. In contrast to M. tuberculosis, the wild-type L. pneumophila phagosome rapidly clears MHC class I molecules and excludes MHC class II molecules as well as all endosomal-lysosomal markers studied. In contrast to phagosomes containing either live M. tuberculosis or live wild-type L. pneumophila, phagosomes containing heat-killed M. tuberculosis, live avirulent mutant L. pneumophila, or polystyrene beads stain intensely for lysosome-associated membrane glycoproteins and cathepsin D.

These findings suggest that M. tuberculosis retards the maturation of its phagosome along the endosomal-lysosomal pathway and resides in a compartment with endosomal, as opposed to lysosomal, characteristics. In addition, these findings demonstrate that the intraphagosomal pathway followed by several intracellular parasites that inhibit phagosome-lysosome fusion is heterogeneous.

B3-014 PROGRESS IN THE DEVELOPMENT OF A SUBUNIT VACCINE AGAINST TUBERCULOSIS AND A NEW NONHUMAN PRIMATE MODEL OF PULMONARY TUBERCULOSIS, Marcus A. Horwitz[1], Byong-Wha E. Lee[1], Barbara Jane Dillon[1], Günter Harth[1], Esterlina V. Tan[2], Eduardo C. Dela Cruz[2], Rodolfo M. Abalos[2], Jerome B. Nazareno[3], Leon J. Young[2], Laarni G. Villahermosa[2], and Gerald P. Walsh[2], [1]Department of Medicine, University of California, Los Angeles, [2]Leonard Wood Memorial, Cebu, The Philippines, and [3]SICONBREC, Manila, The Philippines.

A safe and effective subunit vaccine against tuberculosis would have important advantages over Mycobacterium bovis BCG, the only vaccine currently available. Mycobacterium tuberculosis is an intracellular parasite, and in previous studies, we have demonstrated the importance of extracellular proteins of intracellular pathogens in inducing cell-mediated immune responses and protective immunity against this group of pathogens. We now have explored the immunoprotective capacity of purified major extracellular proteins of M. tuberculosis in the guinea pig model of pulmonary tuberculosis, a model noteworthy for its relevance to human disease. We purified the 12 most abundant proteins to homogeneity from culture supernatant fluid. We then immunized guinea pigs intradermally with the proteins in SAF adjuvant and challenged the animals with a large aerosol dose of the highly virulent Erdman strain of M. tuberculosis. Compared with sham-immunized (adjuvant only) controls, guinea pigs immunized with the 30 kDa major secretory protein, alone or in combination with other extracellular proteins, were protected against weight loss and death during the observation period, and at necropsy, they had less lung pathology and fewer viable M. tuberculosis in their lungs and spleens.

Nonhuman primate models of tuberculosis are invaluable for evaluating prototypic vaccines. The rhesus monkey has been used, but maintaining rhesus colonies in the U.S. is very expensive. In contrast, the cynomolgus monkey (Macaca fasicularis) can be maintained and studied in the Philippines at relatively low cost, prompting us to study its potential as a model for tuberculosis. To do so, we challenged 12 born-in-captivity monkeys in groups of 4 with 10^3, 10^4, or 10^5 colony forming units (CFU) of M. tuberculosis Erdman strain by intratracheal instillation into the right mainstem bronchus, and monitored the animals clinically and immunologically. All animals developed tuberculosis. Monkeys receiving doses of 10^5 or 10^4 CFU developed bilateral pneumonia that was rapidly fatal with mean survival times of 31 and 63 days, respectively. At necropsy, these animals exhibited hilar and mediastinal lymphadenopathy, lung consolidation, caseation necrosis, Langhans giant cells, and some animals had miliary spread to the liver, spleen, and kidneys. Animals receiving 10^3 CFU developed a slowly progressive disease manifested by abnormal chest x-ray findings (right lower lobe pneumonia) by 4 weeks, elevated erythrocyte sedimentation rate by 5 weeks, and weight loss by 13 weeks. The disease remained confined to the right lung until 12 weeks after challenge. Animals in this group consistently demonstrated positive lymphocyte proliferative responses to PPD after challenge, but skin-test responses to PPD-S were only intermittently positive. Mean survival time exceeded 19 weeks.

We conclude that a) purified extracellular proteins of M. tuberculosis show promise as a subunit vaccine against tuberculosis and b) cynomolgus monkeys develop pulmonary tuberculosis that clinically, pathologically, and immunologically resembles human tuberculosis.

B3-015 HOST AND BACTERIAL DETERMINANTS THAT MEDIATE PHAGOCYTOSIS OF M. TUBERCULOSIS, Larry S. Schlesinger, Thomas M. Kaufman, Byoung K. Kang, and Laura K. Marchiando, VA Medical Center and The University of Iowa, Iowa City, IA 52242.

A hallmark of tuberculosis infection is the ingestion of *Mycobacterium tuberculosis* (*M.tb*) by host mononuclear phagocytes followed by intracellular multiplication. Unique molecules on the bacterial surface that bind to specific phagocyte receptors mediate phagocytosis and may dictate the fate of intracellular bacteria. Our laboratory has identified several major receptors on human mononuclear phagocytes that mediate phagocytosis of two well-characterized laboratory virulent strains (Erdman and H37Rv) and the attenuated H37Ra strain of *M.tb*. While phagocyte complement receptors mediate phagocytosis of both virulent and attenuated strains, the macrophage mannose receptor (MR) is also important in phagocytosis of the virulent strains. Thus use of the MR during bacterial entry may be important in enhancing survival of bacteria in phagocytes. We have determined that the major surface lipoglycan, lipoarabinomannan (LAM), from the Erdman strain (ManLAM) is a ligand for the MR via its terminal mannosyl units. We have developed a model in which LAM from several *M.tb* species is bound to hydrophobic polystyrene microspheres to study the interaction between phagocytes and LAM further. Microspheres coated with ManLAM, H37RvLAM, H37RaLAM and LAM from an avirulent mycobacterium (AraLAM) are incubated with monocytes and monocyte-derived macrophages in the absence of serum (LAM and anti-LAM antibodies provided by Patrick Brennan and colleagues, NIH contract N01-AI25147). ManLAM microspheres (ManLAM-M) demonstrate a 3-fold increase in adherence to macrophages compared to AraLAM-M and control-M that is abolished by down-modulation of the MR and by anti-MR antibody. To differentiate between attachment and ingestion of ManLAM-M, phagocytes containing microspheres are fixed with either methanol or formalin prior to indirect immunofluorescent staining of microspheres for LAM to assess total cell-associated and attached microspheres, respectively. These studies indicate that for macrophages, after a short period of attachment of ManLAM-M, there is a rapid ingestion phase that continues to increase linearly over time. There is a low steady state level of attachment over this time period. The level of attachment is markedly reduced and the level of ingestion is abolished at 4°C (evidence for energy dependence), in the presence of the dihydrocytochalasin B (evidence for cytoskeleton dependence) and in the presence of EDTA (evidence for divalent cation-dependence). These findings are consistent with receptor-mediated endocytosis of ManLAM-M and with involvement of the MR. In contrast to macrophages, monocytes demonstrate minimal adherence of ManLAM-M. Preliminary electron microscopy studies indicate that essentially all ManLAM-M are intracellular, confirming fluorescence microscopy results. H37RvLAM-M and H37RaLAM-M demonstrate intermediate levels of adherence to macrophages. Soluble mannan inhibits adherence of ManLAM-M by 70±7%, H37RvLAM-M by 48±14%, H37RaLAM-M by 23±14%, AraLAM-M by 8±19% and control-M by -11±10%. Preliminary ELISA studies suggest significant differences in antigenic specificities among LAM species providing further evidence for subtle differences in the structure of LAMs that may influence interactions with the host cell. The goal of these studies is to relate structural differences among LAM species to their function and will provide further insight into the role of a major mycobacterial surface lipoglycan in early host cell interactions.

Immunoregulation in Tuberculosis

B3-016 SPECIFIC RECOGNITION OF MYCOLIC ACID ANTIGENS BY T LYMPHOCYTES. Evan M. Beckman, Steven A. Porcelli, Craig T. Morita, Samuel M. Behar, Stephen Furlong and Michael B. Brenner. Lymphocyte Biology Section, Dept of Rheumatology/Immunology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115.

MHC class I and class II molecules present peptide antigens to αβ T cells. An analogous antigen presenting function has been proposed for the non-MHC encoded CD1 molecules, expressed on most professional antigen presenting cells. We have previously demonstrated that CD1 molecules are recognized as ligands by certain autoreactive $CD4^-CD8^-$ T cells and isolated multiple examples of αβ T cells restricted by either CD1b or CD1c to foreign microbial antigens. Although associated with β2-microglobulin, the nonpolymorphic CD1 glycoproteins have little direct sequence homology with MHC class I or class II molecules and are predicted to have a different secondary structure in their α2 domains. This suggested that the antigens presented by CD1 might be different from peptide antigens bound by MHC molecules. To determine the chemical nature of the antigens presented by CD1 molecules, we purified the CD1b restricted antigen recognized by one representative CD4-CD8- αβ T cell line from *Mycobacteria tuberculosis* sonicates. The protease resistant and hydrophobic CD1b restricted antigen was purified by rpHPLC and shown to be mycolic acid, a family of long chain α-branched, β-hydroxy fatty acids unique to mycobacteria and several closely related species. The recognition in this system is not the result of a mitogenic response, as other mycobacteria specific T cells not restricted by CD1b do not respond to purified mycolic acid. The divergence of CD1 structure from that of MHC molecules and the nature of the antigens recognized by this system, indicate that CD1 antigen presentation should be viewed as separate from MHC encoded presentation. Moreover, the CD1 restricted recognition of nonprotein antigens enlarges the potential repertoire of antigens recognized by αβ T cells. Finally, these results suggest that the unique lipids of mycobacteria may be important in the T cell immune response to mycobacterial infections.

B3-017 REGULATION OF THE HUMAN CELLULAR IMMUNE RESPONSE IN TUBERCULOSIS, Jerrold J. Ellner, Case Western Reserve University, Cleveland, OH 44106-4984

Pulmonary tuberculosis is characterized by activation of local and systemic immune and inflammatory cells and mediators. Cross-modulatory cytokines, produced by mononuclear phagocytes, chiefly transforming growth factor-beta (TGFβ) deactivate effector function, depress T-cell activation and may account for increased antibody levels. Ingestion of *M. tuberculosis* stimulates expression of cytokines. Tumor necrosis factor-alpha (TNFα) and TGFβ function in autocrine circuits producing activating and deactivating signals respectively for macrophage effector function. In TB patients, TGFβ is expressed in granulomas, and blood monocytes show increased spontaneous and tuberculin PPD-stimulated production of this cytokine. Moreover, neutralizing antibody to TGFβ reverses depressed PPD-stimulated blastogenesis. Therefore, TGFβ appears to be a key mediator of immunosuppression and anergy. Increased local and systemic TGFβ may partly reflect the preserved capacity of lipoarabinomannans from virulent *M. tuberculosis* to induce expression of this cytokine. In dually-infected persons, activation of CD4 lymphocytes and monocytes by TB may promote replication of human immunodeficiency virus-Type 1 (HIV). In fact, serum and cell-derived TNFα are increased in HIV-infected TB patients and this cytokine is known to stimulate HIV replication. Further, monocytes from TB patients show increased infectability by HIV, and PPD and *M. tuberculosis* stimulate HIV replication in latently infected macrophages. The human immune response can be used to identify candidates for a subunit vaccine. Increasing evidence supports a primary role for extracellular products of *M. tuberculosis* as a target of protective immunity. Epitope mapping of the secreted 30kD alpha antigen indicates immunodominance of certain peptides that could provide the basis for an improved vaccine or DTH skin test.

B3-018 THALIDOMIDE TREATMENT REDUCES TNF-ALPHA PRODUCTION AND ENHANCES WEIGHT GAIN IN PATIENTS WITH PULMONARY TUBERCULOSIS. Gilla Kaplan. The Rockefeller University, New York, NY 10021.

Chronic enhanced production of TNF-alpha is associated with pathological manifestations of both HIV-1 and M.tuberculosis infections. These include cachexia, fevers, malaise, anorexia and muscle weakness. High levels of TNF-alpha have been reported in patients with AIDS. It has been suggested that the accelerated progression to full blown AIDS observed in HIV-1 positive patients co-infected with M.tuberculosis results from excess TNF-alpha production. Monocytes isolated from tuberculosis patients with active disease and systemic symptoms release enhanced amounts of TNF-alpha into the culture supernatant in response to stimuli and non-stimulated freshly isolated monocytes from tuberculosis patients constitutively express TNF-alpha mRNA. TNF-alpha has also been shown to activate HIV-1 replication in vitro.

We have shown that thalidomide selectively inhibits TNF-alpha production in vitro and in vivo. Therefore, thalidomide therapy of tuberculosis patients might reduce levels of TNF-alpha and associated toxicities linked with overproduction of the cytokine, mainly TNF-alpha induced cachexia. This should occur without interfering with the immune response against the bacillus.

We have conducted a double-blind placebo controlled clinical study with thalidomide. The drug was given to patients with tuberculosis and HIV-1 associated wasting for up to 21 days at the dose of 300 mg/day. The drug was found to be safe and well tolerated. A significant thalidomide induced weight gain was observed, in addition to general symptomatic improvement. Thalidomide therapy did not result in immunosuppression, as assessed by delayed type hypersensitive response (DTH) skin testing. In patients co-infected with M.tuberculosis and HIV-1 higher levels of TNF-alpha were found, as compared to patients infected with a single pathogen. A highly significant correlation between plasma TNF-alpha levels and plasma HIV-1 titers was observed in these patients. Thalidomide therapy was associated with a reduction in plasma TNF-alpha levels. The effect of thalidomide treatment on HIV-1 viral titers is under investigation.

B3-019 REGULATION OF VACCINE-INDUCED RESISTANCE IN A GUINEA PIG MODEL OF PULMONARY TUBERCULOSIS, David N. McMurray, Elsie S. Mainali, Margaret S. Smith and Susan W. Phalen, Department of Medical Microbiology and Immunology, Texas A&M University Health Science Center, College Station, TX 77843-1114.

Resistance to pulmonary infection with virulent *Mycobacterium tuberculosis* in guinea pigs was modified by vaccination, dietary restriction, or pharmacological alteration of the cytokine environment. Successful vaccination with *M. bovis* BCG resulted in the development of CD4+ T cell populations which were capable of migrating into inflammatory exudates induced by specific antigens (e.g. PPD, killed mycobacteria), producing high levels of IL-2, proliferating extensively, activating macrophages to inhibit the intracellular growth of virulent tubercle bacilli *in vitro*, and transferring a high level of resistance to syngeneic naive recipients. Vaccinated animals produced small, well-circumscribed, highly-organized pulmonary granulomas. In contrast, the induction of chronic, moderate dietary protein deficiency coincident with BCG vaccination resulted in shifts of CD4 and CD8 T cell populations from the circulation into lymph nodes draining the infection site, inability to mobilize T cells into antigen-specific inflammatory exudates, the loss of antigen-induced proliferation and IL-2 production *in vitro*, and the ineffective adoptive transfer of protection to naive recipients. Protein-deprived, vaccinated guinea pigs developed diffuse, poorly-defined granulomas in the lung. Tumor necrosis factor (TNF) was produced in response to antigenic stimulation both in inflammatory exudates *in vivo* and in cell culture *in vitro*. The suppression of TNF production by thalidomide was not associated with an alteration in disease resistance. Immune complexes containing mycobacterial antigens down-regulated the *in vitro* proliferative response of lymphocytes to both ConA and PPD in the presence of Fcγ receptor-bearing lymphocytes, but only in cultures of cells from normally-nourished guinea pigs. Preliminary attempts to induce nitric oxide (NO) formation in guinea pig macrophages by exposure to combinations of mycobacteria, interferon gamma (IFNγ) and LPS were unsuccessful. Taken together, these data suggest that tuberculosis resistance in this experimental model depends upon the ability of the guinea pig to mobilize antigen-reactive, IL-2-producing CD4T cells into infectious foci where they can activate macrophages and generate mature granulomas. High levels of circulating immune complexes are likely to be immuno-suppressive in the infected guinea pig. (Supported, in part, by USPHS, NIH grants AI-15495 and AI-27204).

Pathophysiology of Tuberculosis in the Lung

B3-020 EXOCHELINS OF MYCOBACTERIUM TUBERCULOSIS: CHARACTERIZATION AND DEMONSTRATION OF THEIR CAPACITY TO REMOVE IRON FROM HOST IRON-BINDING PROTEINS, Jovana Gobin[1], Christopher Moore[2], Joseph R. Reeve Jr[1], Diane Wong[2], Bradford W. Gibson[2], and Marcus A. Horwitz[1], [1]Department of Medicine, University of California, Los Angeles, and [2]Department of Chemistry and Pharmaceutical Chemistry, University of California, San Francisco.

To multiply and cause infection, Mycobacterium tuberculosis must acquire iron from the host. To do so, it releases small water soluble siderophores called exochelins. Exochelins are thought to transfer iron to another type of iron-binding molecule located in the bacterial cell wall, the mycobactins, for subsequent utilization by the bacterium.

To characterize exochelins, we have purified these molecules for the first time, and analyzed them by mass spectrometry. Exochelins comprise a family of molecules whose major species range in mass from 744 to 800 daltons in the neutral Fe^{3+}-loaded state. The molecules form two 14 Da-increment series, one saturated and the other unsaturated, with the increments reflecting different numbers of CH_2 groups on a side chain. Based on a comparison of their tandem mass spectra, exochelins and mycobactins share a common core structure. However, exochelins are smaller than mycobactins due to a shorter alkyl side chain, which terminates, in the case of exochelins, in a methyl ester. These differences render exochelins more polar than the lipophilic mycobactins and hence more soluble in the aqueous extracellular milieu of the bacterium in which they bind iron in the host.

To clarify the role of exochelins in the infected host, we have studied their capacity to compete for iron with host iron-binding proteins in vitro. We converted purified exochelins to their desferri form and incubated these with human transferrin, lactoferrin, or ferritin at 4:1 and 1:1 molar ratios of iron:exochelin. We then assayed the conversion of desferri-exochelins to ferri-exochelins at various times by reverse-phase HPLC. Within one minute of incubation with 95% iron-saturated transferrin, desferri-exochelins acquired iron from transferrin, and by 1 hour, the exochelins in the reaction vessel were fully iron-saturated. No conversion of desferri-exochelins to ferri-exochelins was detected when desferri-exochelins were incubated with apotransferrin. Desferri-exochelins also readily removed iron from 40% iron-saturated transferrin, the approximate percent saturation of this protein in human serum. Similarly, desferri-exochelins acquired iron from iron-saturated lactoferrin and ferritin, although acquisition of iron from ferritin was slower than from the other iron binding proteins.

This study provides the first compositional and structural characterization of exochelins and demonstrates that purified exochelins of M. tuberculosis have the capacity to remove iron from three high-affinity iron-binding molecules of the human host - transferrin, lactoferrin and ferritin. This study thus supports the hypothesis that, at extracellular sites of M. tuberculosis multiplication in the host, such as lung cavities, where free iron is very limited, the bacterium utilizes exochelins to acquire iron from host iron-binding proteins.

B3-021 HISTOPATHOLOGICAL CONSEQUENCES OF LUNG INFECTION WITH *M. TUBERCULOSIS* IN IMMUNOCOMPETENT AND IMMUNOINCOMPETENT MICE, Robert J. North and Pamela L. Dunn, Trudeau Institute, Saranac Lake, NY.

Immunity to infection with *Mycobacterium tuberculosis* (Mtb) is a classical example of T cell-mediated immunity to infection. According to the results of adoptive immunization and T cell depletion studies in mice, immunity is mediated predominantly by $CD4^+$ T cells, although $CD8^+$ T cells play a role. The $CD4^+$ T cells that mediate immunity are of the T helper-1 type, in that the involvement of IFN-γ is essential. This type of immunity is generated in response to infection with virulent, as well as vaccine, strains of Mtb. However, immunity to vaccine strains is successful at resolving infection in all organs, whereas immunity to virulent strains is not. In the latter case infection persists in the lung to cause progressive disease. This was confirmed by a recent study in this laboratory which compared the manifestations of virulence in mice of several strains of Mtb and *M. bovis* in terms of the ability of the strains to grow in the lungs and other organs and to kill their host. It was found in all cases that intravenous inoculation of a standard number (10^5) of bacilli eventually resulted in slowly progressive infection in the lungs, in the face of an acquired state of systemic $CD4^+$ T cell-mediated immunity capable of almost completely resolving and then stabilizing infection in other organs. It is apparent from examining published studies from other laboratories and from the results of experiments recently performed in this laboratory that the inability to resolve infection in the lungs also applies to mice vaccinated with BCG. A histological study of the lungs of mice infected via the intravenous route showed that during the first 50 days of so post inoculation, each focus of infection had induced an extensive mononuclear pneumonitis that occupied a large number of contiguous air sacs. The dominant cells were large epithelioid macrophages many of which contained acid-fast bacilli. At this stage of infection the basic architecture of the lung in these lesions was essentially intact, in spite of consolidation caused by the intraalveolar build up of macrophages. With the passage of time, however, the histopathology of the lesions become increasingly chronic in nature. By day 300 each lesion had expanded to a considerable size and had fused with other lesions to form large areas of dense fibrosis that completely replaced most of the original lung structure. Mtb was not present in this fibrous tissue, but was located in macrophages at its leading edges. At the time mice began dying, they possessed only residual lung structure and function, although the exact cause of death is not known. It is this late chronic type of inflammation that is more relevant to the disease in humans, rather than the earlier more acute type that is the subject of most publications dealing with this subject. Chronic lung inflammation did not have time to develop in the lungs of immunoincompetent mice infected with the same number of Mtb. In these mice lung inflammation was more acute, more expansive, and more destructive, because of unrestricted growth of the bacterium and the types of inflammatory cells that were attracted.

Epidemiology and Diagnosis of Tb; Application of Molecular Probes

B3-022 AMPLIFICATION ASSAYS FOR DIAGNOSIS OF TUBERCULOSIS, Kathleen D. Eisenach, Ph.D., University of Arkansas for Medical Sciences, Pathology Department, Little Rock, Arkansas.

A dramatic improvement in the rapidity and accuracy of laboratory testing for *Mycobacterium tuberculosis* is anticipated in the near future from the application of molecular biology techniques. The polymerase chain reaction (PCR) and alternative nucleic acid amplification methods have the potential to detect small numbers of tubercle bacilli in a clinical specimen within hours of collection. Most amplification systems described thus far are specific for *M. tuberculosis*, however, several have the ability to detect all mycobacteria. Sequences of the IS6110, 16S rRNA gene, and 16S rRNA have been widely used as targets for amplification. In the near future two commercial systems will be available in the US. One of these is the PCR-based Roche Molecular Systems AMPLICOR MTB Test that targets the 16S rRNA gene. The other is the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test that is based on the isothermal amplification of target rRNA by DNA intermediates. Initial studies indicate that these tests are highly specific and the sensitivities range from 71% to 100%. Results with these tests are similar to those obtained with research-based assays (homebrew PCR). Sensitivity tends to be lower with samples that contain low numbers of tubercle bacilli. The lower limit of detection is effected by the efficiency of the lysis method and the overall sample processing.

Most studies have compared these assays to culture and smear, using the microbiology as the "gold standard". Since the assays have the potential to be more sensitive than culture, results should be compared to the clinical diagnosis. These assays may also be useful in determining the bacterial load during the course of therapy. Studies are needed to evaluate this application.

Other systems for detecting *M. tuberculosis* are under development. These are based on the following methodologies: strand displacement amplification (SDA) (Becton Dickinson), Q-beta replicase amplification (Gene-Trak), ligase chain reaction (LCR) (Abbott), signal amplification branched-DNA (Chiron), and cycling probe technology (CPT) (ID Biomedical).

In our laboratory we are working with the IS6110 PCR assay, the research-based SDA assay that amplifies a sequence within IS6110, and the CPT assay that uses probes complementary to the 6-bp direct repeat. Ongoing studies involving these assays will be described.

B3-023 MOLECULAR APPROACHES TO THE DIAGNOSIS OF TUBERCULOSIS, Thomas M. Shinnick, Centers for Disease Control and Prevention, Atlanta, GA 30333.

The timely identification of persons infected with *Mycobacterium tuberculosis* and the rapid laboratory confirmation of tuberculosis are two key ingredients of effective public health measures to combat the resurgence of tuberculosis and the recent outbreaks of nosocomially transmitted multidrug-resistant tuberculosis. Until recently, the standardly used procedures for detecting *M. tuberculosis* in patient specimens started with microscopic examination of smears for the presence of acid-fast bacilli followed by culturing of the organisms on solid media and then biochemical tests on the cultured organisms to identify which *Mycobacterium* species was present. Drug susceptibility tests were usually done after the identification step. This process often required 6 to 8 weeks from the time of specimen collection to provide species identification and susceptibility results, primarily because of the slow growth of mycobacteria. The use of liquid media and the BACTEC radiometric culture system can shorten this process to about 4 weeks, and it can be further shortened by using rapid molecular diagnostic procedures for the identification step, such as nucleic acid probes, and by plating portions of the patient specimens directly onto drug plates for susceptibility testing. Current CDC recommendations for diagnostic laboratory performance are 1) process specimens daily, even on weekends, 2) inoculate liquid media (e.g., BACTEC) for the primary culture, 3) use nucleic acid probes or the NAP test to identify growth as *M. tuberculosis* as soon as possible, 4) determine drug susceptibility using liquid media, and 5) report results of each step to physicians in a timely manner. The immediate goals are to report identification of *M. tuberculosis* within 10 to 14 days and drug susceptibilities within 15 to 30 days.

New molecular approaches to identifying *M. tuberculosis* and determining drug susceptibilities have been described recently. Gene-amplification techniques and luciferase-reporter-phage systems may be able to reduce the time required for determining drug susceptibilities from 2 to 3 weeks to 1 to 2 days. Several amplification-based systems for the identification of *M. tuberculosis* are nearing the marketplace and may soon enable a clinical laboratory to report the confirmation of an *M. tuberculosis* infection to the physician within hours of receiving a specimen.

Late Abstract

LIGASE CHAIN REACTION: A NEW MOLECULAR NON-CULTURE METHOD FOR THE DIRECT DETECTION OF TUBERCULOSIS, Helen H. Lee, Gregor L. Leckie, Jianli Cao, Hsiang-Yun Hu and Omar Khalil, Probe Diagnostics Business Unit, Diagnostics Division, Abbott Laboratories, Abbott Park, IL 60064

Ligase Chain Reaction (LCR) is a technique which amplifies specific nucleic acid sequences. 4 single-stranded probes are designed such that probes 1 and 3 are complementary to the 3' and 5' halves respectively of the plus target strand while probes 2 and 4 to the minus strand. Amplification is achieved by repeated cycles of heating and cooling for strand separation and specific hybridization. Amplicons are formed via ligation of adjacent probes. To reduce target independent ligation, gap-LCR was developed in which the ends of the oligonucleotide probes are positioned such that, upon hybridization, a gap of several bases exists. The gap between adjacent probes is subsequently filled by the appropriate nucleotides using DNA polymerase. Amplicons are detected via immuno-capture by labeling the ends of each probe pair with different capture and detection haptens. Carry-over contamination of amplicons is prevented by chemical inactivation of amplicons post detection. In addition, amplicon detection is carried out in a closed automated system in which reactions are manipulated by piercing the amplification tube.

An LCR DNA amplification assay for direct detection of *Mycobacterium tuberculosis* (MTB) in respiratory specimens processed for culture has been developed. The total assay time is less than 6 hours with a sensitivity of detecting 5 genomes of MTB DNA. DNA from 138 MTB clinical isolates from 4 different geographical regions were tested, all were positive. The specificity of the probe set has been validated by testing DNA from 45 mycobacterial species and 10 non-mycobacterial species. A total of 400 respiratory specimens from 6 different sites were processed for LCR testing. The MTB LCR assay detected 66/70 smear positive culture positive specimens, 17/20 smear negative culture positive specimens. In addition, 24/310 smear negative culture negative specimens tested positive by LCR, all of which were confirmed by a probe set against a different gene region. Using an expanded gold standard consisting of culture positive and confirmed LCR positive samples, the MTB LCR assay has a sensitivity and specificity of 93.8 and 100% respectively, while the equivalent figures for culture were 78.9% and 100%. Approximately 2% (7/400) of the specimens contained substance(s) which inhibited LCR reaction. Progress needs to be made in sample preparation to remove inhibition to further improve LCR sensitivity.

Structure, Biogenesis and Targeting of the Cell Wall of M. tuberculosis; The Proteins of M. tuberculosis

B3-100 POST-TRANSLATIONAL GLYCOSYLATION OF THE *MYCOBACTERIUM TUBERCULOSIS* 19-KDA PROTEIN, Christiane Abou-Zeid[1], Ying Zhang[1], Douglas B. Young[1], Karen M. Dobos[2], Delphi Chatterjee[2], kay-Hooi khoo[2], and Patrick J. Brennan[2], [1]Department of Medical Microbiology, St. Mary's Hospital Medical School, London W2 1PG, and [2]Department of Microbiology, Colorado State University, Fort Collins, CO 80523.

Proteins that are secreted by live *M. tuberculosis* are important targets of the immune response and represent attractive candidates for inclusion in subunit vaccines. Some of these proteins are subject to post-translational modifications which may affect their immunogenicity. We have investigated the use of a rapid-growing mycobacteria *M. vaccae* to express the *M. tuberculosis* 19-kDa antigen which is secreted across the cell membrane and modified by glycosylation and acylation. Using the hygromycin resistance vector we obtained efficient expression of the 19-kDa protein (50-100 fold higher than in extracts of *M. tuberculosis*). The recombinant 19-kDa bound strongly to concanavalin A. The 19-kDa protein was purified from both filtrate and cell extracts of the recombinant *M. vaccae* by fractionation with ammonium sulphate followed by anion-exchange and size exclusion chromatography, and RP-HPLC. Analysis of the sugar content by gas chromatography/mass spectrometry revealed that the secreted form of the 19-kDa protein was 10% glycosylated by mannose mainly. Glycopeptides are currently being isolated by proteolytic digestion of the 19-kDa protein to determine the glycosylation sites and the structure of the glycosyl moieties. The findings of this study will be helpful to establish optimal ways to produce secreted antigens of *M. tuberculosis* and to understand the immune response to mycobacteria.

B3-101 **SURFACE PROPERTIES OF CORD FACTOR**, Rami Almog and Carmen Mannella, Division of Molecular Medicine Wadsworth Center for Laboratories and Research, New York State Department of Health. P.O. Box 509 Albany, New York 12201

Systematic investigation of the surface properties of synthetic cord factor (trehalose 6,6 -dimycolate), containing mycolic acid residues isolated from M. tuberculosis (gift of M.B. Goren, National Jewish Hospital, Denver, CO), was performed using the monolayer technique at the air-aqueous interface. The area occupied by one molecule in a closed - packed orientation (A_{cp}), and the "liftoff" area per molecule when change in Π is first detected, (A_{lo}), were obtained from surface pressure - molecular area (Π-A) isotherm plots (n=12). The values were 161 ± 7 Å^2/molecule and 316 ± 36 Å^2/molecule, respectively. Compression-expansion-recompression data showed that the cord factor molecules on recompression had the same A_{cp} (162 ± 8Å^2/molecule), but significantly ($p < .0001$) smaller "liftoff " area (A_{lo} = 198 ± 26Å^2/molecule). Furthermore, at Π = 10 mN/m, the cord factor monolayer had significantly ($p < .0001$) larger surface compressional modulus (SCM) during recompression (103 ±18 mN/m) than in the first compression (69 ± 8 mN/m). The change in Π with time at constant area showed that cord factor monolayer is not stable below 5mN/m. However, the collapse surface pressure was relatively stable, suggesting that cord factor formed lamellar structures. The Π-A plot of the first compression is typical of a "liquid-expanded" monolayer. The SCM is considerably smaller than that of saturated phospholipid (160 mN/m); it is characteristic of loose lateral packing, which indicates poor steric accommodation of the long hydrocarbon chains of the mycolic acid residues. The chains must reorient during the first compression, allowing tighter packing and increased SCM on recompression, resulting in a "liquid-condensed" monolayer.

B3-102 *Abstract Withdrawn*

B3-103 DETECTION OF PROTEIN ANTIGENS EXPRESSED BY A *M.BOVIS* - BCG COSMID LIBRARY AND ANALYSIS OF THE CORRESPONDING GENES , Marion Bona, Rabindranath Nayak, Maria Mincek, Lisa Pascopella, William R. Jacobs and Jerrold J. Ellner, Case Western Reserve University, Cleveland, OH., Indian Institute of Science, Bangalore, India and Albert Einstein College of Medicine, New York, N.Y.

Detection of novel mycobacterial protein antigens expressed from a genomic library in a homologous system has been hampered by the number of crossreacting antigens, whereas cloned mycobacterial genes are only inefficiently expressed in a heterologous system. We have developed a novel approach to screen an *M.bovis*-BCG cosmid library expressed in *M. smegmatis* for immunologically active protein antigens. The method detects *M.bovis*-BCG specific antigens, reducing interference of crossreacting antibodies. Duplicate western blots of culture filtrate proteins were incubated with (a) hyperimmune rabbit serum directed against *M.bovis*-BCG antigens and (b) the same serum, but preadsorbed with a *M.smegmatis* cell lysate. Comparing these duplicate western blots allowed differentiation between true and false positives. 31 of 350 cosmids, expressing *M.bovis*-BCG specific antigens were thus identified. 5 of these were secreted in sufficient quantity to be identified by gold staining. Testing of the 31 positives with a panel of monoclonal antibodies targeting similarly-sized antigens resulted in detection of the 19 kDa antigen expressed by cosmid 147. None of the candidates reacted with the panel of monoclonal antibodies. The inserts of cosmids 129, 156 and 223, expressing new antigens of approximately 100 kDa were subcloned. The coding sequence for the antigen expressed by cosmid 223 was located on a 5 KB Bam H1 fragment. These subclones will be used to identify the genes and characterize their products.

B3-104 IDENTIFICATION OF A HEPARIN-BINDING MYCOBACTERIAL HEMAGGLUTININ. Michael J. Brennan*, Maryline Laude-Sharp*, Julie H. Hannah*, Franco Menozzi#, and Camille Locht#. *DBP, CBER, FDA, Bethesda, MD 20852 and #Microbiologie Genetique et Moleculaire, Institut Pasteur, 59019 Lille Cedex, France. In an effort to identify potential adhesins expressed by mycobacteria, proteins were purified by heparin-Sepharose chromatography from BCG culture filtrates and eluted fractions were tested for hemagglutination. Fractions containing a 27/28 kDa doublet on SDS-PAGE agglutinated rabbit red blood cells and Western blot analyses indicated that these proteins differ from known fibronectin-binding proteins. This hemagglutinin was quantitatively isolated from membrane enriched preparations of *M. tuberculosis* H37Rv, H37Ra and BCG using heparin-Sepharose chromatography. Monoclonal antibodies (MAbs) directed against this heparin-binding protein (HBP) recognize the 27/28 kDa doublet and diffuse higher molecular weight material of 55-65 kDa, indicating that an oligomeric complex may be present. Immunogold-electron microscopy using the MAbs demonstrated that the HBP is a major surface antigen on H37Ra. In a novel assay, the addition of 0.5 µg/ml of HBP to dispersed H37Ra results in aggregation of the bacteria. This aggregation can be inhibited by 2.5 µg/ml of the MAb and by sulfated sugars. These studies suggest that the HBP may be involved in bacterial-bacterial interactions and possibly in lectin-like interactions with eukaryotic cells.

B3-105 MYCOBACTERIAL PROTEIN ANTIGENS: ISOLATION AND CHARACTERISATION OF 35 kDa MAJOR MEMBRANE PROTEIN (MMPI) OF *MYCOBACTERIUM LEPRAE* AND *MYCOBACTERIUM AVIUM*. Britton WJ, Winter N, Triccas JA, Rivoire B*, Pessolani MCV*, Brennan PJ*.
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The development of vaccines against mycobacterial infections requires the characterisation of major immunogenic proteins for different mycobacteria. These may be shared by different mycobacteria or have a limited distribution. Physical purification of proteins from the non-cultivable species, *M. leprae*, identified a 35 kDa major membrane protein (MMPI) which stimulated *M. leprae* specific antibody responses in leprosy patients. We have utilised a PCR strategy with primers based on the sequence of peptides derived by proteolytic clearance from MMPI to isolate the *mmPI* gene from an *M. leprae* cosmid library. This encoded a protein of 307 amino acid with predicted Mr of 33.8 kDa, slightly less than the mass of 34.5 kDa as determined by ES mass spectroscopy of the native protein. rMMPI expressed in *E. coli* reacted with mAb raised to the native protein MMPI has no apparent homology with published protein sequences, however, a hydrophobic region (287-300) at the C-terminus contains a possible transmembrane spanning domain. Southern blot analysis revealed that the *mmPI* gene was absent from members of the *M. tuberculosis* complex, but present in isolates of *M. avium* and *M. haemophilium*. Sequence analysis of *M. avium mmPI* showed 82% DNA and 85% amino acid identity with *M. leprae* MMPI. The identification of MMPI in a cultivable mycobacterium will facilitate functional studies of this protein. When the *mmPI* gene was expressed in the fast growing *M. smegmatis* under the control of an "up" regulated promoter from *M. fortuitum*, there was enhanced production of soluble MMPI. After purification by mAb affinity chromatography, the *M. smegmatis* - derived rMMPI was more immunogenic than *E. coli*-derived protein.

B3-106 STRUCTURE AND GENETIC ORGANIZATION OF A PHOSPHATE PERMEASE FROM *M. TUBERCULOSIS*, Jean Content, Martine Braibant, Priska Peirs, Lucas De Wit, Philippe Lefèvre, Åse Anderson* and Kris Huygen. Department of Virology, Pasteur Institute Brabant, Brussels, B-1180 Belgium.*, Staten Seruminstitut Copenhagen, DK-2300, Denmark.

Antigen 88 is the immunodominant B cell antigen in BCG for H-2^b mice infected with live BCG. It is highly homologous to PstA, one of the two internal membrane proteins from the *E. coli* phosphate permease operon (Braibant et al. *Inf. Immun.* 1994, 62, 849-854). We now present the structure of two other subunits of the *M. tuberculosis* phosphate permease: the second internal membrane component (the PstC homolog) and the ATP-binding subunit (PstB homolog) that have been cloned and sequenced. These genes have been mapped within a genomic region including the genes coding for Ag88 and for the previously described antigen pab (the presumed equivalent to *E. coli* PstS or PhoS: Andersen and Hansen, *Infect. Immun.* 1989, 57, 2481-88). We have also found two genes homologous to the *E. coli* PstS and one additional ORF similar to the *E. coli* PstC within this same genomic region. Functional studies of the phosphate permease in *M. bovis* BCG and *M. smegmatis* and expression of the mycobacterial phosphate permease operon in *E. coli* have been undertaken to enable the screening of potentially inhibitory drugs and of monoclonal antibodies. Finally we have recently identified the gene encoding an eukaryotic type of serine-threonine protein kinase (MbK) in *M. tuberculosis* and in *M. bovis* BCG, within the (putative) phosphate permease operon. This kinase is purified from *M. bovis* BCG and its biochemical characterization is in progress. Attempts are made to define its specific substrates and its role in clump formation, invasion of cells, virulence, interference with host signal transduction, and regulation of the phosphate permease genes. The characterization of these 4-6 permease subunits and of the MbK kinase could contribute to the knowledge of new biochemical pathways used by mycobacteria, which could lead to definition of new metabolic targets and the design of alternative therapeutic drugs.

B3-107 AN INVESTIGATION OF THE ENDONUCLEASE ACTIVITY OF THE MYCOBACTERIUM TUBERCULOSIS RECA INTEIN, Elaine O. Davis, Peter J. Jenner, Patricia C. Brooks, Farahnaz Movahedzadeh and M. Joseph Colston, Laboratory of Leprosy and Mycobacterial Research, National Institute for Medical Research, Mill Hill, London NW7 1AA, England.

The RecA proteins of *Mycobacterium tuberculosis* and *M. leprae* are produced from a large precursor protein by protein splicing in which an intervening sequence, the intein, is removed and the two flanking regions are ligated to form the mature RecA protein. Inteins have also been discovered in the vacuolar ATPase of the yeast *Saccharomyces cerevisiae* and in the DNA polymerase of the archaebacteria *Thermococcus litoralis* and *Pyrococcus* species and these inteins have been shown to possess site-specific endonuclease activity. Motifs of the pattern LAGLIDADG which are conserved amongst homing endonucleases and are important for endonuclease activity are identifiable in these intein sequences including those of the mycobacteria. Therefore the aim of this study was to establish whether the *M. tuberculosis* intein has endonuclease activity and if so to characterise it. The intein was expressed under the control of a regulatable promoter in *Escherichia coli* for purification. Two types of constructs were used, one in which the intein would splice out of the protein produced, and one in which a tag would be joined to the amino terminus of a modified intein lacking the amino terminal amino acid. The inteins produced by these clones were then purified by ion-exchange and/or affinity chromatography. The yeast and archaebacterial inteins have been shown to bind and cut the DNA sequence around the point of insertion of the intein encoding sequence and in yeast to so mediate homing of the intein encoding DNA. Therefore, a precise deletion of the intein encoding DNA was made in the *M. tuberculosis recA* gene as the most probable substrate for the mycobacterial intein. This construct was used to demonstrate endonuclease activity of the purified intein.

B3-108 GENE CLONING, OVEREXPRESSION AND BIOCHEMICAL ANALYSIS OF THE RIBONUCLEASE HI FROM *MYCOBACTERIUM SMEGMATIS*, Stephanie S. Dawes[1], Robert J. Crouch[2] and Valerie Mizrahi[1]. [1]Molecular Biology Unit, South African Institute for Medical Research and Department of Haematology, University of the Witwatersrand, Johannesburg, South Africa; [2]National Institute of Child Health and Human Development, NIH, Bethesda, MD. 20892, USA.

Activity gel analysis of cellular extracts from slow- and fast-growing mycobacterial species confirmed the presence of several RNA·DNA hybrid-specific RNase activities in both classes of organisms. The *rnhA* gene encoding the RNase HI from *M. smegmatis* was subsequently cloned using a PCR-generated internal gene segment probe (Mizrahi, V. *et al.* (1993) *Gene* **136**, 287-290). In contrast, the cloning method was unsuccessful in identifying the *rnhA* gene in *M. tuberculosis*, presumably as a result of conservative amino acid substitutions within the consensus sequences used for the design of the PCR primers. The *M. smegmatis rnhA* gene has a G+C content of 69% and the deduced polypeptide sequence of 159 amino acids shares 52% amino acid identity with the RNase HI from *E. coli*; significantly, all the active site residues associated with RNase HI function are conserved in the mycobacterial homologue. Unlike its counterparts from *E. coli* and *S. typhimurium*, the mycobacterial gene does not form an overlapping, divergently arranged transcriptional unit with the *dnaQ* gene encoding the ε proofreading subunit of DNA polymerase III. The mycobacterial RNase HI was overexpressed in *E. coli* as a maltose binding protein fusion protein which was purified to homogeneity by affinity chromatography and enzymatically characterized. The fusion protein was functional *in vitro* as evidenced by activity gel analysis of induced cell lysates.

B3-109 CRYSTAL STRUCTURE OF InhA FROM *Mycobacterium tuberculosis*: THE MOLECULAR BASIS OF ISONIAZID RESISTANCE, Andrea Dessen*, Annaik Quemard#§, William R. Jacobs, Jr.#§, and James C. Sacchettini*, Departments of Biochemistry*, Microbiology and Immunology#, and Howard Hughes Medical Institute§, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York, 10461

The emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis* severely compromises worldwide disease control efforts and necessitates the need for novel agents to treat tuberculosis infections. Recently, isoniazid resistance in *M. tuberculosis* has been linked to the expression of a mutant form of InhA, an enzyme most likely to be involved in mycolic acid biosynthesis. The three-dimensional structures of both isoniazid-sensitivity and isoniazid-resistance conferring forms of InhA from *M. tuberculosis* have been determined and the geometries of the active sites suggest that drug resistance is directly related to a perturbation in the hydrogen-bonding network which stabilizes cofactor binding. This region of the protein is involved in drug resistance in both clinical and laboratory MDR-TB isolates. The availability of the structures of both sensitive and resistant forms of this known isoniazid target is a powerful tool for future rational anti-turbercular drug design approaches.

B3-110 GALACTOSE-1-PHOSPHATE URIDYLTRANSFERASE ACTIVITY IN *MYCOBACTERIUM SMEGMATIS*, Ken Duncan, David Monsey, Pam Nassau and Anthony Weston, Glaxo Research and Development, Stevenage, Herts, UK

Glycoside biosynthesis typically involves the formation of activated sugar nucleotide molecules. The Leloir pathway of galactose metabolism yields one such nucleotide, UDP-galactose, which may be an important intermediate in the biogenesis of the arabinogalactan portion of the mycobacterial cell wall. The enzymes which are responsible for synthesising the cell wall components make attractive targets for novel anti-TB chemotherapeutic agents. As a model system, we are studying galactose metabolism in *M. smegmatis*. A cell-free extract of *M. smegmatis* was prepared and used to synthesise UDP-galactose from galactose-1-phosphate and UDP-glucose. The enzyme which catalyses this reaction is galactose-1-phosphate uridyltransferase. A Pharmacia Resource Q anionic exchange column was used to purify the enzyme 20-fold, with concomitant separation from UDP-galactose-4-phosphate epimerase activity. Crude extracts did not contain UDP-galactose pyrophosphorylase activity. Analysis of the newly synthesised UDP-galactose (following phosphodiesterase treatment and Dionex HPLC) revealed that >99% of the galactose residues released by this method were in the pyranose form. Conversion of the galactose moiety of UDP-galactose into the furanose form must occur at a later stage. This is the first time that galactose-1-phosphate uridyltransferase has been demonstrated in *M. smegmatis*.

B3-111 BIOSYNTHESIS AND POLYMERIZATION OF D-ARABINOSE IN MYCOBACTERIA, Alan D. Elbein, Andrea Wang and Y.T. Pan, Department of Biochemistry and Molecular Biology, Univ. of Arkansas for Medical Sciences, Little Rock, AR 72205.

D-Arabinose (ara) is a major sugar of mycobacteria since it is found in the cell wall arabinogalactans and arabinomannans of these organisms. D-ara is an unusual component of complex carbohydrates, and only occurs in mycobacteria and a few related bacteria, and in some lower eucaryotes. However, it is not found in humans. Thus, steps in the biosynthesis or polymerization of D-ara should be excellent target sites for chemotherapy, assuming that the arabinogalactan and/or arabinomannan are essential to the viability of these organisms. Thus, we have initiated studies to define the pathway of D-ara activation and polymerization in Myco. smegmatis. Nothing is currently known about how this sugar is formed in living cells, but the following are possibilities for its biosynthesis: D ara could arise via the pentose-P pathway from ribose-5-P, or it could be formed as a result of decarboxylation of a hexose-P or nucleoside-diphosphate hexose, or it could be synthesized by the condensation of 2 and 3 carbon units. Methodology was developed in our lab to separate D-ara from other pentoses and hexoses on the carbohydrate analyzer, and other methods to fractionate cells and separate sugar-Ps, nucleoside diphosphate sugars and polyprenyl-linked sugars. M. smegmatis was then incubated with various labeled sugars (^{3}H-Man,^{3}H-Gal,^{3}H-Glc,^{3}H-Xyl) and fractionated to give rise to the various intermediate compounds referred to above. ^{3}H-Gal was the best precursor of D-ara. However, in the presence of ^{3}H-Man or ^{3}H-Gal radioactive D-ara was found in the nucleoside diphosphate sugar fraction and in the lipid fraction. The radioactivity in D-ara in the lipid fraction and in the cell wall was abolished when cells were incubated in ^{3}H-Gal in the presence of ethambutol. The radioactive D-ara containing compounds are being further analyzed to characterize the lipids and nucleotides.

B3-112 GENE CLONING AND PURIFICATION OF PROTEINS SECRETED BY *Mycobacterium tuberculosis*. Maria Laura Gennaro, Claudia Manca and Donatella Usai, Public Health Research Institute, New York, NY 10016.

The long-term goal of our studies is to identify protein antigens actively secreted by *M. tuberculosis* that elicit protective T cell responses and are potentially useful for production of subunit vaccines.

To obtain *M. tuberculosis* genes expressing secreted proteins, we screened an *M. tuberculosis* recombinant phage expression library with antibodies directed against secreted proteins (kindly provided by several investigators as well as by the WHO Monoclonal Antibody Bank at the Centers for Disease Control, Atlanta, GA). Using polyclonal antibody preparation K12 given to us by Dr. H.G. Wiker (University of Oslo, Norway), we have isolated a phage clone expressing a 18-Kda protein of *M. tuberculosis* (that should presumably correspond to the secreted protein MPT63, whose coding sequence was previously unknown). Following subcloning of the antigen-coding region, we have initiated nucleotide sequencing of this novel gene. We are also in the process of cloning this coding sequence in a recombinant gene expression vector that allows high-level expression as well as simple one-step protein purification. The purified antigen will be characterized in terms of T-cell responses by immunologists collaborating to this project (Drs. S. Demaria and Y. Buskin, PHRI).

A similar approach, based on library screening with antibodies as well as PCR technology, is being used to clone other known genes expressing *M. tuberculosis* secreted proteins. Several clones have been obtained that are currently being characterized. Preparation of yet unknown antigens from culture filtrates of *M. tuberculosis* is also in progress.

B3-113 CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* GLUTAMINE SYNTHETASE, Günter Harth, Daniel L. Clemens, and Marcus A. Horwitz, Department of Medicine, School of Medicine, University of California, Los Angeles, CA 90024

The development of new strategies to combat tuberculosis requires more knowledge about *M. tuberculosis* molecules that mediate pathogenesis including host cell-pathogen interaction. In this regard, we have identified the mycobacterial glutamine synthetase as one important protein and purified the enzyme to apparent homogeneity from three-week old culture filtrates. The native enzyme has a molecular weight of 680,000 Da consisting of 12 most likely identical subunits of 58,000 Da. The enzyme subunits are arranged in two hexagonally shaped ring structures, one stacked upon the other. The 20 N-terminal amino acids of the enzyme are highly homologous with other members of the glutamine synthetase family.

The enzyme catalyzes the formation of glutamine from glutamate, ammonia, and ATP, a reaction that requires manganese ions and a pH optimum of 7.0 - 7.5. The enzyme is relatively resistant to heat treatment and degradation by proteases, but is rapidly inactivated by denaturing or chelating agents and by its specific inhibitor L-methionine-*S*-sulfoximine, which also inhibits growth of *M. tuberculosis* in broth cultures.

M. tuberculosis continuously releases up to 30% of the total measurable glutamine synthetase activity into its culture medium, where all enzyme substrates and reaction products are detected. Comparisons with other mycobacterial species revealed that only pathogenic species exhibit release of the enzyme into the culture medium. *M. tuberculosis* also releases the enzyme into its phagosome in infected human monocytes, providing the first example of a mycobacterial protein released into the phagosomal space.

Our study suggests two potentially important roles for glutamine synthetase in the pathogenesis of *M. tuberculosis* infection. First, the release by pathogenic mycobacteria of active glutamine synthetase into the growth medium indicates that the enzyme is directly involved in the extracellular synthesis of glutamine, a major component of the cell wall of pathogenic mycobacterial species. Second, the presence of glutamine synthetase in the phagosomal space of *M. tuberculosis* may influence ammonia concentrations within the phagosome, and play a role in the organism's capacity to inhibit phagosome-lysosome fusion and phagosome acidification in host cells.

B3-114 **CLONING AND EXPRESSION OF THE CELL DIVISION GENE *ftsX* OF *Mycobacterium tuberculosis*,** Asha K. Kinger and Jaya S. Tyagi, Department of Biotechnology, All India Institute of Medical Sciences, New Delhi-110 029, INDIA.

Mycobacterium tuberculosis, causative agent of human tuberculosis, is characterized by long cell generation times with doubling time of ~20h. Growth within macrophages is vital for the survival and spread of *M. tuberculosis*. Nothing is known at the genetic level about the factors that control cell division in mycobacteria. We report the identification and partial characterization of a putative cell division gene *ftsX* from *M. tuberculosis* ($ftsX_{Mt}$). Using a subtracted cDNA probe, genes that are differentially expressed in *M. tuberculosis* H37Rv (*dev*) were identified and cloned (Kinger and Tyagi, 1993, 131, 113-117). Sequence analysis of one of the *dev* clone, pAK51 revealed an open reading frame (ORF) that encodes putative protein with an M_r of 32,800 which showed 65.8% homology with the cell division protein FtsX from *Escherichia coli*. The expression of recombinant $FtsX_{Mt}$ from a T7-based vector inhibited the growth of *E. coli*. Southern hybridization studies indicated that $ftsX_{Mt}$ gene is conserved among slow- and fast- growing mycobacteria suggesting an essential function for it. mRNA transcripts derived from $ftsX_{Mt}$ gene were detected in *M. smegmatis* by RT-PCR and their abundance was proportional to the different stages of growth. The value of this gene as a marker to evaluate mycobacterial growth will be discussed.

B3-115 Summary of CDC Guidelines for the Prevention of *Mycobacterium tuberculosis* transmission in health-care facilities. Linda S. Martin, CDC TB Guidelines Work Group, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Atlanta, GA 30333

Transmission of *M. tuberculosis* is a recognized risk to patients and health care workers (HCWs) in health-care facilities. More than 100 HCWs have had tuberculin skin test conversions; at least 17 have developed active multi-drug resistant TB (MDR-TB); and at least 6 have died from outbreaks of MDR-TB. Designated personnel should conduct a risk assessment for the entire facility and implement an appropriate control program. Levels of control measures are based on the classification of risk for a facility, specific ares, or for a specific occupational group. This risk classification is based on the profile of TB in the community, the number of infectious TB patients admitted or where area, and the results of analysis of HCW purified protein derivative (PPD) test conversions and possible person-to-person transmission of *M. tuberculosis*. Early recognition and treatment of infection of *M. tuberculosis* and tuberculous disease are vital to reduction of transmission of *M. tuberculosis*.The CDC has revised the guidelines to emphasize the importance of the hierarchy of control measures, including administrative and engineering controls and personal respiratory protection; health-care facility risk assessment and development of a written tuberculosis (TB) control plan; early detection and management of persons who have TB, PPD skin-testing programs; and HCW education. Engineering controls functions primarily by isolating the hazard from the worker. Guidelines for laboratory personnel working with *M. tuberculosis* have been updated. Key components of the guidelines will be featured.

B3-116 THE ORIGIN OF THE CARBON ATOMS OF CELL WALL ARABINOSE IN MYCOBACTERIA, Michael McNeil, Michael Schermann, Lingyi Deng, Luise Bournonville, Duane Bush and Patrick Brennan, Department of Microbiology, Colorado State University, Fort Collins, CO 80523

A long term goal of our research is to develop new drugs that inhibit mycobacterial cell wall D-arabinan biosynthesis. However, nothing is known about biosynthetic origin of the D-arabinose carbon atoms. Possible pathways include 1) C-6 decarboxylation from hexuronic acids, 2) the oxidative decarboxylation of C-1 from glucose-6-P (oxidative leg of the pentose shunt), 3) the non-oxidative pentose shunt pathway (i.e. 5 fructose-6-phosphate →6 pentose phosphates), or 4) novel condensations from triose to pentose. To obtain information on this issue mycobacteria were fed glucose labeled in various positions. The cell walls were isolated, hydrolyzed, the arabinose and galactose separated by HPLC and the radioactivity in each sugar determined. Glucose labeled at C-6 or glucose labeled at C-1 were converted to cell wall arabinose equally well. These results ruled out "hexose" decarboxylation at either C-6 or C-1. Further experiments showed that the cell wall arabinose was readily labeled using radioactive ribose as a precursor suggesting the arabinose carbon skeleton was made via the pentose shunt but, at least under the growth conditions used, by the non-oxidative pathway. Experiments to determine the position of the labeled atoms in the mycobacterial synthesized arabinose are on going and these results are expected to shed further light on the metabolic origin of this important cell wall component.

B3-117 STUDIES ON THE DNA PRIMASE FROM MYCOBACTERIUM TUBERCULOSIS.

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DNA primases are essential in organisms that undergo bidirectional DNA replication: at a replication fork, primases initiate DNA synthesis on the displaced lagging strand by synthesizing a short oligoribonucleotide primer *de novo*. Studies on the DNA primase of bacteriophage T7 have demonstrated that the T7 primase contains a conserved zinc motif that is required for primase activity. Interactions between the primase and the primase recognition sequence, 3'-CTG-5', on single stranded DNA template rely on an intact zinc motif in the amino terminus of the T7 primase. At primase recognition sequences, 3'-CTG(G/T)(G/T)-5', T7 primase synthesizes primarily tetraribonucleotides of the sequence pppAC(C/A)(C/A). Thus, the T7 primase requires site-specific DNA-protein interactions to activate the primase polymerase activity. Prokaryotic primases may also use a similar mechanism to catalyze primer synthesis. For example, the *Escherichia coli* DnaG primase is a zinc metalloprotein with a potential zinc binding motif in the amino terminus of the protein. The DnaG protein preferentially initiates primer synthesis from the trinucleotide sequences 3'-GTC-5', where in a fashion similar to that of the T7 system, the 3'-nucleotide of the recognition sequence is conserved yet not used as a template during oligoribonucleotide catalysis. We are examining the catalytic mechanism of DNA primases of several Mycobacterial species and report our progress in the isolation and characterization of the gene for the *M. tuberculosis* DnaG primase. Since the enzymatic mechanisms of prokaryotic and eukaryotic DNA primases appear to be distinct, prokaryotic DNA primases represent a potential novel target to inhibit the growth of bacterial pathogens such a *M. tuberculosis*.

B3-118 FURTHER STUDIES ON EFFECTS OF ETHAMBUTOL ON METABOLISM OF MYCOBACTERIUM SMEGMATIS,

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The effectiveness of ethambutol (EMB) as an antituberculosis drug has been known for about 30 years. There is evidence that EMB affects biosynthesis of some of the cell wall components of *M. tuberculosis;* however, the exact molecular mechanism of EMB action is not known. The effects of EMB on the synthesis of cell wall arabinogalactan (AG), lipid, LAM and LM were investigated in EMB-susceptible and EMB-resistant strains of *M. smegmatis.* In EMB-treated cells, a rapid and specific inhibition of ^{14}C glucose incorporation into the arabinan portion of arabinogalactan as well as the accumulation of trehalose-dimycolate was observed. In contrast, EMB-resistant strain did not reveal either of these effects. Analysis of LAM from EMB-treated cells also showed a dramatic inhibition of its synthesis whereas incorporation into LM was increased, again pointing to a specific inhibition of arabinan synthesis. Interestingly, an EMB-resistant strain, grown in the presence of EMB, produced a smaller, truncated form of LAM with decreased Ara content. Since LAM is a key factor in the survival of *Mycobacterium* within phagocytic cells and AG is a key structural component, the inhibition of arabinan biosynthesis seems to be a crucial event in the bacteriostatic action of EMB. The results provide us with new directions in development of novel chemotherapeutic agents against TB. [Supported by a grant (AI-30189) from the NCDDG-OI program, NIAID, NIH].

B3-119 APPROACH TO ISOLATION OF *M. SMEGMATIS* MUTANTS DEFECTIVE IN PROTEIN GLYCOSYLATION

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The 19 kD antigen of *M. tuberculosis* has been found to be associated with carbohydrate in its purified form, suggesting that it may be a glycoprotein. Glycoproteins are thought to be rare in prokaryotes, and little is known about the nature of the carbohydrate or its putative attachment to the protein backbone. In order to study this process we have cloned the 19 kD antigen promoter and coding sequence into pOLYG19, a mycobacterial shuttle vector which confers hygromycin resistance. When the resulting plasmid (pDM1) is expressed in *M. smegmatis* mc^2155, the 19 kD antigen produced is of similar size to the native form seen in *M. tuberculosis*, and also has associated carbohydrate as determined by concanavalin A staining after SDS-PAGE analysis of culture supernatants or cell extracts. In order to isolate mutants defective in associated carbohydrate, we have developed a sandwich ELISA assay utilizing lectins in the capture phase. The mutant *M. smegmatis* library pCG79::mc^2155 (Guilhot, C. J. Bacteriol. 176:535-9) was transformed with pDM1, creating a library of mutants expressing the 19 kD antigen. Individual mutants are screened by making cell lysates, "capturing" the 19 kD antigen by lectins bound to the bottom of microtiter plate wells, and then detecting the presence of the antigen by the monoclonal antibody IT-10. This strategy is designed to identify mutants lacking in the capacity to glycosylate protein substrates, resulting in a decreased signal in the ELISA phase of the assay. The use of these mutants to identify enzymes that effect glycosyl linkages in mycobacterial proteins may contribute to the development of new drug targets.

B3-120 **IDENTIFICATION OF A NOVEL 30 KDA ANTIGEN OF M. TUBERCULOSIS (MTB)**

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Several antigenic components of Mtb have been identified. Some of these components have been localised at the cellular level, with an aim to establish its accessibility to the host immune response. In our laboratory we have identified an immunoreactive 30 kDa protein antigen of Mtb (H37Rv). In order to localise the antigen at the cellular level, three methods were used (1) Immunoblotting (2) ELISA & (3) Immuno-electron microscopy (IEM). Monospecific sera to the antigen was generated in mice. For immunoblotting, sonic extracts & culture filtrate of MTB (4 ug/ul) were fractionated on SDS-PAGE gradient gels (5-20%). The fractionated antigens were electro blotted on to nitrocellulose. The electroblots were then probed with the murine monospecific sera (1:200) and exposed to anti-mouse AP conjugate (1:1000) followed by the substrate (BCIP & NBT). Intact bacilli ($5x10^5$/well) and equivalent amounts of the sonic extract were coated in ELISA Plates. The ELISA plates were exposed to control and immunized murine sera over range of dilutions and followed by anti-mouse HRP conjugate (1:1000). The appropriate substrate was added and plates read at 490 nm. For IEM, the bacilli derived from actively growing cultures, were heat killed, fixed and embedded in resin. Ultrathin sections of bacilli (50 nm) were exposed to pre-immunized and immunized sera. The binding of the antibody was detected using protein A gold conjugate (1:50).

The immunoblots showed that the antigen was associated with sonic extracts of MTB and was absent in the culture filtrates. In the ELISA reactivity of the immune sera was observed at all dilutions with the MTB sonicate exclusively. The electron micrographs showed the concentration of the gold conjugates in the cytoplasm and inner membrane of the mycobacterial cell.

These results demonstrate the direct evidence of the intracellular location of the 30 kDa antigen in *Mycobacterium tuberculosis*. Hence differs from the reported secretory antigens of MTB in a similar molecular weight range.

B3-121 **STUDY OF NAD PATHWAY OF MYCOBACTERIUM TUBERCULOSIS**

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None of the biosynthetic pathway of **Mycobacterium tuberculosis (Mtb)** has been caracterized at the molecular level. A better understanding of such a pathway may identify new targets for antituberculosis drugs. As a part of a project to examine the NAD pathway of **Mycobacterium tuberculosis,** we initiated a study to caracterize the gene encoding quinolinate synthetase (QS) , an enzyme in the novo pathway.

We constructed a genomic library of **Mtb** (H37Ra, ATCC 25177) in pBluescript. The recombinant plasmids were transformed into niacin-auxotrophic **E. coli** (PA-2-18) which lacks the nadA gene that encodes the **E. coli** QS. The trans-formed PA-2-18 strains were screened for growth in niacinfree medium.

One recombinant clone PA-2-18(pMH24) was able to grow in the absence of niacin and produce niacin in the medium. The plasmid pMH24 harbored a 2.0 Kb DNA fragment. The recombinant **E. coli** strain PA-2-18 (pMH24) grown in niacin free medium expressed 2 polypeptides with MW of 53 Kd and 28 KD. The sequence analysis of the **Mtb** DNA fragment is being completed. Based on these results so far, we have cloned the QS gene or a gene regulating QS in **Mtb.**

B3-122 MYCOTHIOL AND MYCOTHIOLDISULFIDE REDUCTASE FROM MYCOBACTERIA.

Daniel J. Steenkamp*, Hendrik S.C.Spies^ and Elaine Rumbak* Department of Chemical Pathology, University of Cape Town Medical School* and NMR Laboratory, University of Stellenbosch, South Africa^.

Many gram positive bacteria lack glutathione and produce alternate thiol species, few of which have as yet been characterised. Analysis of perchloric acid extracts of *Mycobacterium bovis* (BCG) by means of the fluorogenic maleimide, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), indicated the presence of a single major thiol species. This compound was assigned the trivial name mycothiol. Mycothiol was isolated, either as the bimane derivative or as the disulfide, by means of a two-step chromatographic procedure. The structure of mycothiol was investigated by one and two-dimensional NMR spectroscopy of the bimane derivative. Spatial relationships within the molecule were established by NOE experiments and allowed the identification of mycothiol as the novel compound, 1-D-*myo*-inositol-2-(N-acetyl-L-cysteinyl)amino-a-D-glucopyranoside. This assignment was further supported by FAB-mass spectrometry which indicated m^+/z = 677.6 as predicted from the proposed structure. Chemical analysis indicated the presence of equimolar glucosamine and cysteic acid. Mycothiol was also identified as the principal thiol of *M.tuberculosis* and *M.intracellulare*. Crude extracts of *M. bovis* contained an enzyme which catalysed $NADPH_2$ dependent reduction of mycothioldisulfide. Mycothioldisulfide reductase has low natural abundance and has thusfar only been partially purified.

B3-123 CLONING OF MYCOBACTERIAL GENES INVOLVED IN CELL WALL BIOSYNTHESIS, Neil G. Stoker, Lucy A. Brooks, David Minnikin[1], and Paul R. Wheeler, Bacterial Molecular Genetics Unit, Department of Clinical Sciences, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK and [1]Department of Chemistry, University of Newcastle, Newcastle-upon-Tyne NE1 7RU, UK

The mycobacterial cell wall is a highly complex structure, which has been implicated in the virulence of the organism, and as a target for novel antibiotics. Much of the structure has been determined, but very little is known about the biosynthesis of the molecules making up the cell wall. We have been using genetic and biochemical approaches to investigate the biosynthetic pathways.

Following chemical mutagenesis we have gridded out a bank of *M. smegmatis* colonies, which has then been screened for a number of phenotypes which may reflect an altered cell wall. We identified a number of colonies which showed increased sensitivity to either pyrazinamide and penicillin, which may be due increased permeability caused by a defective cell wall. We have analysed one of the mutants, Pyramid1, further. This mutant is more sensitive to pyrazinamide than the parent strain, and has a smooth rounded colony morphology. When an *M. leprae* shuttle cosmid library was introduced, a high proportion of transformants regained either the wild-type colony morphology, the pyrazinamide sensitivity, or both. This suggested that more than one *M. leprae* sequence was affecting the *M. smegmatis* Pyramid1 phenotype, possibly by producing enzymes which modified the cell wall. We have started to analyse these *M. smegmatis* clones biochemically, and have identified a novel spot by TLC, supporting the hypothesis that the *M. leprae* DNA in this clone encodes enzymes involved in synthesizing cell wall components. This is a promising approach to determine the functions of clusters of genes from pathogenic mycobacteria, and we are extending it to *M. tuberculosis.*

B3-124 GENETIC AND PHENOTYPIC PROPERTIES OF AN EXCEPTIONAL MYCOBACTERIUM TUBERCULOSIS STRAIN WITH SWITCHING COLONY MORPHOLOGY. Dick van Soolingen[1], Peter Hermans[2], Theo Hoogenboezem[2], Petra de Haas[2], Patrick Brennan[3], Leo Schouls[1], and Jan van Embden[1]. Dept. of LBA and MMB[1], National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven and Dept. of Pediatrics[2], Sophia Childrens Hospital, Erasmus University of Rotterdam, PO Box 1738, 3000 DR Rotterdam, the Netherlands. Dept. of Microbiology[3], Colorado State University, College of Veterinary Medicine and Biomedical Sciences, Fort Collins, Colorado 80523, USA.

We investigated a Somalian *M. tuberculosis* isolate, strain 16778, which is exceptional in growing with smooth colonies on solid medium and which switches to a rough colony morphology with a frequency of about 10^{-4}. In liquid medium this strain was found to grow with a minimal generation time of 14 hours, about 5 to 8 hours less than normally observed for *M. tuberculosis*. In contrast to previously investigated *M. tuberculosis* strains, this isolate harbours only a single IS1081 element, whereas other *M. tuberculosis* strains invariably contain 5 to 7 copies of this insertion sequence. Pulse field gel electrophoresis of XbaI-cleaved chromosomal DNA showed that strain 16778 showed a fragment pattern which is clearly distinct from other *M. tuberculosis* strains investigated so far. Strain 16778 was also exceptional in the presence of only 2 known spacer sequences within the Direct Repeat(DR) locus of the chromosome. Strain 16778 differs from other *M. tuberculosis* strains in the expression of at least 2 proteins as measured by 2-D analysis. No differences in the smooth and the rough variant of strain 16778 were found using a wide variety of genetic markers, and also not by two dimensional gel electrophoresis of total protein. However the rough variant differed from the smooth one in the absence of a particular lipooligosaccharide. These data indicate that the Somalian isolate may belong to a separate genetic grouping of *M. tuberculosis* complex bacteria which has not previously been recognized.

B3-125 CHEMICAL AND BIOLOGICAL PROPERTIES OF LIPOARABINOMANNAN (LAM) BYPRODUCTS PROCESSED BY MURINE ALVEOLAR MACROPHAGES. Mary W. Vermeulen[1], Eugene Oh[1], Jiayi Wu[1], and Matthew J. Fenton[2]. [1]Pulmonary and Critical Care Unit, Massachusetts General Hospital and Harvard Medical School, and [2]Pulmonary Center, Boston University Medical School; Boston, MA

Recent reports from several labs have shown that lipoarabinomannan (LAM) structures purified from mycobacterial cell walls are potent stimulators of mammalian immune responses, including cytokine induction, although the responses have been reported to vary depending on the presence or absence of mannose capping of LAM, and/or the virulence of the organism from which the LAM is isolated. We found that when cells of the AMJ2C11 mouse alveolar macrophage line are incubated with cell walls of *M. bovis* BCG or *M. tuberculosis* H37Ra or H37Rv, TNF is induced in a pattern that differs from that reported for the corresponding purified LAMs. We next investigated whether LAM or similar structures are actual mycobacterial cell wall byproducts of antigen processing by alveolar macrophages. *M. bovis* BCG or *M. tuberculosis* H37Rv were grown with S-[^{14}C-methyl]adenosyl methionine, a precursor in LAM biosynthesis. Resulting cell walls were incubated with AMJ2C11 alveolar macrophages for 48 hours. Culture supernatants were lyophilized, dialyzed, and analyzed for LAM-like catabolites. Gel filtration (Bio-Gel P-100), followed by anion exchange (DEAE-Sephacel) chromatography yielded 7 major fractions from BCG, and 4 from H37Rv. Chemical analyses revealed few differences from synthetically purified LAMs, although biological activities suggested that macrophage processing altered properties of subsequent TNF induction in alveolar macrophages.

Supported by NIH grant RO1 HL51957.

B3-126 ARABINOSE-5-PHOSPHATE METABOLISM IN *MYCOBACTERIUM SMEGMATIS*, Anthony Weston, Andy Whittington, Richard Upton, Ken Duncan, *Lingyi Deng and *Mike McNeil. Glaxo Research and Development, Stevenage, Hertfordshire, and *Colorado State University, Fort Collins, Colorado

Little is known about the origin of the arabinose residues found in mycobacterial cell wall arabinogalactan (AG). Takayama and Kilburn (AAC 33: 1493) recently proposed that arabinose-5-phosphate (A-5-P) is an intermediate in AG biosynthesis, being derived from ribulose-5-phosphate (Ru-5-P) by the activity of an enzyme A-5-P isomerase. It was also proposed that A-5-P was converted to arabinose-1-phosphate (A-1-P) then, via a sugar nucleotide, incorporated into AG. We have tested the hypothesis with cell-free extracts from *M. smegmatis* as a source of the catalytic enzymes, but could find no evidence for the conversion of A-5-P to Ru-5-P when monitored either by the carbazole reaction or by ^{31}P NMR. Furthermore, A-5-P was not converted to either ribose-5-phosphate (R-5-P) or ribose-1-phosphate (R-1-P). The assay techniques used could detect A-5-P isomerase activity in *E.coli* extracts. Crude extracts of *M. smegmatis* were able to convert both R-5-P and R-1-P to keto sugars as shown by a strong positive carbazole reaction. Dionex HPLC and ^{31}P NMR also showed that A-5-P was not converted to either α-A-1-P (furanose form) or α-A-1-P (pyranose form) but was converted to two new peaks as revealed by ^{31}P NMR. One was shown to be inorganic phosphate but the other has still to be characterised. A-5-P was isolated from whole cells and [^{14}C] A-5-P was incorporated into AG in a cell-free system but at a very low level (1.0%). In conclusion, *M.smegmatis* did not have any A-5-P isomerase activity nor was it able to convert A-5-P to A-1-P. Because of this and the low level of incorporation of A-5-P into AG, the present evidence does not favour A-5-P as a normal intermediate in AG synthesis. We are currently considering other possible pathways including conversion of ribose to arabinose at the sugar nucleotide or lipid level.

B3-127 LIPID INTERMEDIATES FOR ARABINOGALACTAN ARABINOMANNAN BIOSYNTHESIS IN MYCOBACTERIA, Beata A. Wolucka, Department of Chemistry, University of Louvain, 1348 Louvain-la-Neuve, Belgium.

Glycosyl-P-undecaprenols are common sugar donors in the biosynthesis of cell-envelope polysaccharides of many bacteria. In mycobacteria, however, phosphorylated forms of two unusual polyprenols (C_{50} decaprenyl-P and C_{35} octahydroheptaprenyl-P) are present instead of the "classical" undecaprenol. Our studies on D-arabinose biosynthesis in mycobacteria led to the discovery of the polyprenyl-P-sugar family containing arabinose, ribose and mannose in *M. smegmatis*. The complete structure of two major pentose-containing compounds, β-D-arabinofuranosyl-1-monophosphodecaprenol and β-ribosyl-1-monophosphodecaprenol, was established by combined GC-MS, FAB-MS MS and ^{1}H-NMR. Mycobacterial C_{50}-decaprenol differs from bacterial C_{55}-undecaprenols not only by its chain length but also by the presence of only one internal *trans*-isoprene residue. Thus, both mycobacterial pentofuranoses, D-Ara and D-Rib are β-linked to the unusual mono-*trans*, octa-*cis* decaprenyl-P. On the contrary, the majority of mannosyl residues of the polyprenyl-P-sugar pool of *M. smegmatis* is attached to the shorter, partially saturated C_{35} octahydroheptaprenyl-P, suggesting that the two mycobacterial polyprenols may have different biological functions. *In vivo* pulse-chase experiments demonstrated that polyprenyl-P-Ara, Rib and Man are rapidly turning-over intermediates, the lipid-ribose and lipid-mannose being the major ones. Moreover, sugar composition of the polyprenyl-P-sugar fraction isolated from *M. tuberculosis* H37Ra and *M. avium* serovar 8 after *in vivo* labeling was very similar to that of *M. smegmatis* indicating that decaprenyl-P-ribose is a common lipid intermediate in mycobacteria. Additionally, treatment of *M. smegmatis* cells with two antituberculosis drugs known to impair the formation of the arabinogalactan-mycolate (AG-M) complex (i.e., ethambutol and isoniazide), resulted in the accumulation of decaprenyl-P-arabinose and the compensative decrease of the decaprenyl-P-ribose content. This effect was very rapid for ethambutol (less than 2 min) and lasted for several hours demonstrating that the drug does not block the conversion of glucose into (lipid-linked) D-arabinose, and suggesting that it inhibits either a specific arabinosyltransferase or the biosynthesis of the D-Ara acceptor itself. In the case of isoniazide known to inhibit mycolic acid synthesis, longer time of exposure was necessary to observe similar changes at the level of the lipid-linked pentoses. To explain the isoniazide effect, it is proposed that mycoloylation may be an obligatory step for arabinogalactan elongation in the biosynthesis of AG-M complex. In the light of the lack of information about ribose-containing polysaccharides in mycobacteria, these findings further support our hypothesis that decaprenyl-P-ribose, through a unique 2'-epimerization, could be a direct precursor of decaprenyl-P-arabinose, a donor of arabinosyl residues for the biosynthesis of the key cell-wall polymers in mycobacteria.

B3-128 MYCOBACTERIAL DNA GYRASE AS A TARGET FOR NEW DRUG SCREENING, Ling-Chuan C. Wu, and S. I. Shahied, Public Health and Environmental Laboratories, New Jersey Department of Health, Trenton, NJ 08652-0360

The emergence of multi-drug resistant strains of *M. tb* has focused attention on the urgent need for new drug development. Of the possible drug targets in *M. tb*, DNA gyrase offers the following advantages: it is phylogenetically different from its mammalian counterparts; it is essential for bacterial survival; its function and mechanism for regulating DNA superhelicity is well-characterized in other prokaryotes; and drugs, such as fluoroquinolones, have been studied as gyrase A subunit inhibitors.

To further investigate the specificity and the sensitivity of quinolone derivatives, the development of cell-free, *in vitro* biochemical assays would be very beneficial. New drugs that target DNA nicking and closing events could be easily screened. To this end, we cloned both the *gyrA* and *gyrB* genes from *M. bovis* BCG using PCR amplification and genomic Southern techniques. We sequenced portions of the *gyrA* gene, and found that the putative active tyrosine binding site and the quinolone resistance determining regions are identical to the *M. tb gyrA* gene. Furthermore, we found that the restriction enzyme cutting sites within the *gyrA* and *gyrB* genes of BCG were identical to those of *M. tb*.

We are in the process of purifying the active enzymes by engineering the genes in proper vectors for high level, single step isolation. In the near future, we expect to demonstrate the Mycobacterial DNA gyrase activity *in vitro* by showing its ability to introduce negative superhelical turns into covalently closed circular DNA in an ATP-dependent manner.

B3-129 THE BIOSYNTHESIS OF CYCLOPROPANATED MYCOLIC ACIDS BY PATHOGENIC MYCOBACTERIA, Ying Yuan, Richard E. Lee, Gurdyal S. Besra, John T. Belisle, and Clifton E. Barry, III, Mycobacterial Research Unit, Rocky Mountain Laboratories, NIAID, Hamilton, MT 59840, and Department of Microbiology, Colorado State University, Fort Collins, CO 80523.

The mycolic acid biosynthetic pathway is an important chemotherapeutic target for the treatment of mycobacterial diseases. Pathogenic mycobacteria modify their mycolic acids by cyclopropanation while saprophytic species do not suggesting that this modification may play a role in protection of these lipids from host oxidative defenses. We have demonstrated the transformation of a *cis*-double bond in the major mycolic acid of *Mycobacterium smegmatis* to a *cis*-cyclopropane ring upon introduction of cosmid DNA from *M. tuberculosis*. The resulting hybrid mycolate possesses features of both saprophytic and pathogenic strains. This activity was localized to a single ORF encoding a protein product of 288 amino acids. This protein is 34% identical to the cyclopropane fatty acid synthase from *E. coli* and therefore represents the second such enzyme to be characterized. Southern blotting demonstrated that *cma1*-homologous sequences were common to pathogenic strains of *M. tuberculosis*, *M. avium* and *M. marinum* while non-pathogenis species such as*M. smegmatis* contain no such sequences. Adjacent regions of the DNA sequence appear to encode ORFs which display significant homology to other enzymes involved in fatty acid metabolism suggesting that some of the enzymes required for mycolic acid biosynthesis may be clustered in this region. The DNA sequence for *cma1* is 55% identical to an unidentified, unpublished sequence from the *M. leprae* genome sequencing project. We have used non-homologous regions from the overlap of these two genes to generate a probe for this gene from *M. leprae*. We have cloned the *M. tuberculosis* homolog of this gene which has been designated *cma2* based upon its probable role in the construction of the second cyclopropane moiety in the full length mycolic acids of pathogenic mycobacteria. Transcriptional regulation of *cma1* and *cma2* will be described as well as the phenotype of *cma*-overproducing strains of *M. smegmatis*. These enzymes, in addition to providing valuable biosynthetic clues in the mycolate pathway, represent novel targets for anti-mycobacterial chemotherapy.

B3-130 A NOVEL LIPOPROTEIN FROM *MYCOBACTERIUM BOVIS* BCG WITH MARKED SEQUENCE SIMILARITY TO THE SECRETED PROTEIN MPB70, Harold Zappe, Wilna Vosloo, Linda Stannard, Patrick Tippoo, Jane Hughes, Dave Beatty and Lafras Steyn, Department of Medical Microbiology, University of Cape Town, Observatory, Cape Town, 7925, South Africa.

We have identified a protein (p23) in *Mycobacterium bovis* BCG (Tokyo) that is expressed at the cell surface. The cloned gene shows a similarity of more than 70% with the *M. bovis* specific, secreted protein MPB70. The similarity, however, is limited to the 3'-end of the genes. This similarity is also reflected in the deduced amino acid sequences of the proteins. The putative signal sequences of the two proteins differ with that of p23 showing the typical features of a prelipoprotein. This feature explains why we are not able to detect any secreted p23 in bacterial cell culture.

In order to produce large quantities of p23 for further studies, we have constructed a maltose binding protein - p23 gene fusion. Interestingly the fusion protein is translocated in *E. coli* cells. Electron micrographs of the *E. coli* cells show the presence of the fusion protein in the cell wall. In addition, there is proteolytic cleavage of the fusion protein in the *E. coli* cells. The cleavage product is smaller than the native p23.

Preliminary indications are that other members of the Genus Mycobacterium contain a similar sized protein that cross-reacts with a monoclonal antibody to p23.

Genetics of M. tuberculosis; Mechanisms of Drug Resistance in Tuberculosis

B3-200 **ALLELIC EXCHANGE IN *M.SMEGMATIS* AND *M. BOVIS* BCG.** V. Balasubramanian, Jean Martin, Kyung Sun-Um, William. R. Jacobs, Jr. Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, N.Y. 10461.

In order to understand the biology of mycobacteria, particularly the molecular pathogenesis, genetic systems allowing the site directed mutagenesis, recombination and complementation are essential. Genetic studies on mycobacteria are at present greatly hampered due to the lack of a powerful, specific and efficient method of mutagenesis. Whereas the ability to perform allelic exchanges on the chromosomes has yielded a useful method of site directed mutagenesis in other organisms, in the clinically important species of Mycobacteria, such as *M. tuberculosis* and *M. bovis*, similar approaches have been as yet unsuccessful. In this study we report the development of a shuttle mutagenesis strategy that involved the use of large linear fragments to reproducibly obtain allelic exchange in *M. smegmatis* and *M. bovis* BCG.

A new cosmid library was constructed in the vector pYUB328, in which the 35-45 kb mycobacterial insert is flanked by two *Pac* I restriction sites; sites which are rare if any in the mycobacterial chromosome. Candidate cosmids were subjected to mini-Tn5 (kanamycin) mutagenesis in *E.coli* and then the *Pac* I liberated insert was used as substrate for recombination. Using this method, gene replacement was achieved in the *inhA* locus of *M.smegmatis*. Random mini-Tn5 insertions within the cosmid encoding the *inhA* locus were obtained and the co-transformation frequecies for the kanamycin and isoniazid resistance were determined.

Gene replacement was performed in *M.bovis* BCG and *M.tuberculosis* in the leucine operon, thereby yielding respective leucine auxotrophs.

B3-201 SIGMA FACTORS OF M.tuberculosis RNA POLYMERASE, T.S. Balganesh, M.Balganesh and Umender K. Sharma, Astra Research Centre India, P.B. No. 359, 18th Cross Malleswaram, Bangalore 560 003, India.

The σ subunit of prokaryotic RNA polymerases confers promoter specific transcription by the core enzyme. The identification, characterisation and sequence analysis of many sigma factors have revealed that they fall into two broad classes. One family is similar to the originally identified E.coli σ^{70} subunit, while the other is similar to the σ^{54} subunit. A complex interplay of different sigma factors regulate transcription of a variety of genes signalled by the physiological needs of the organism.

Mycobacterium tuberculosis is a pulmonary pathogen multiplying intracellularly within alveolar macrophages, also invades extrapulmonary tissues and in addition has a `dormant' state. The sigma factors contributing to the regulated gene transcription of this pathogen have not been identified.

Towards identification and characterisation of genes encoding `sigma' homologues in M.tuberculosis, we have screened M.tuberculosis chromosomal DNA for regions homologous with the highly conserved C-terminal regions of reported σ^{70} sequences. A PCR fragment of ~500 bp was amplified from the chromosome of M.tuberculosis DNA using degenerate sequences homologous to E.coli σ^{70}. Southern hybridisation identified the presence of 3 distinct fragments in the genome of M.tuberculosis H37Rv and H37Ra and only 2 from the genome of M.smegmatis.

Cloning and sequencing of 2 of the hybridising fragments confirmed them to be homologous of the σ^{70} of E.coli. Further characterisation of the physiological role of these σ^{70} homologues is in progress.

B3-202 DETERMINATION OF THE ORIGIN OF REPLICATION OF PLASMID pLR7 FROM *MYCOBACTERIUM AVIUM* LR113. Marjorie L Beggs, Donald Cave and Kathy D. Eisenach, Departments of Pathology and Anatomy, University of Arkansas Medical Sciences Campus, Little Rock, AR 72205

pLR7 is a plasmid that is commonly found in species of *M. avium*. We have been able to identify a fragment of this plasmid that is able to support replication in *M. avium*, *M. tuberculosis* and *M. bovis*. This fragment is 3634 bp in size and has been sequenced and analyzed. It contains a large open reading frame that encodes a 39.9 kd rep protein. Interruption of the reading frame of this protein results in loss of replicative ability. The region adjacent to the rep protein exhibits features characteristic of other plasmid origins. The pLR7 origin of replication shows significant homology to pMSC262 but no homology to pAL5000. This plasmid origin was used to create a new mycobacterial shuttle vector.

B3-203 **MycDB, an integrated mycobacterial database.**

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As part of ongoing efforts to investigate the molecular biology of the human pathogens in the genus *Mycobacterium*, a customised database was developed specifically for these organisms and implemented in ACEDB database manager software. The data loaded include the IMMYC Antigen list, details of reagents available from the CDC/WHO Antibody bank, more than 1 Mbp of sequences of mycobacterial genes and proteins from public databases, the physical maps of *M. leprae* and *M. tuberculosis* developed at the Institut Pasteur, as well as a subset of the references found in MedLine. The ACEDB software allows both quick and intuitive access to the data and to connections between facts by a simple mouse-driven interface, as well as by more powerful query mechanisms.

B3-204 IDENTIFICATION OF STREPTOMYCIN- AND RIFAMPICIN-RESISTANT MUTANTS OF *M. smegmatis* AND IDENTIFICATION OF MUTATIONS BY TEMPERATURE GRADIENT GEL ELECTROPHORESIS. Gordon Churchward*, Teresa Kenney*, Yi-Yi Yu*, Sandra Powell**, and Roger Wartell**,
* Department of Microbiology and Immunology, Emory University, Atlanta, GA 30322 and **School of Biology, Georgia Institute of Technology, Atlanta, GA 30332.

To determine the molecular basis of drug resistance in mycobacteria, two collections of spontaneous mutants of *M. smegmatis* resistant to streptomycin or rifampicin were isolated. Using genetic complementation experiments, the streptomycin resistant mutants were shown to affect the *rpsL* gene of *M. smegmatis*. This gene encodes a ribosomal protein. DNA fragments containing either the *rpsL* gene (streptomycin-resistant mutants) or a segment of the *rpoB* gene (rifampicin-resistant mutants) were amplified from chromosomal DNA isolated from the mutants, and the nucleotide sequence of the amplified DNA fragments was determined. A majority of the streptomycin-resistant mutants were caused by one of four single nucleotide changes that affected two codons. A majority of the rifampicin-resistant mutations contained one of two single nucleotide changes affecting a single codon. In both cases similar mutations cause resistance in other organisms. To develop a system to detect drug-resistant mycobacteria, the melting behavior of fragments from the *rpsL* and *rpoB* genes was analyzed theoretically to select appropriate sequences so that wild type and mutant DNA fragments produced by PCR amplification would show different migration during temperature gradient gel electrophoresis. All mutant fragments tested behaved as expected during electrophoresis and could be distinguished from wild type fragments.

B3-205 EFFECT OF *INHA* AND *KATG* ON ISONIAZID RESISTANCE AND VIRULENCE OF *MYCOBACTERIUM BOVIS*, Desmond M.Collins, Theresa M. Wilson and Geoffrey W.de Lisle, AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand.

Isoniazid (INH) resistance of the *Mycobacterium tuberculosis* Complex which includes *Mycobacterium bovis* is associated with both loss of catalase activity and mutation of the *inhA* gene. However, the relative contributions of these changes to resistance and to the loss of virulence for guinea pigs, that is a feature of many INH resistant strains, is unknown. In this study, a virulent strain of *M. bovis*, was exposed to increasing concentrations of INH. Two INH-resistant strains were produced which had lost catalase activity. Strain WAg405, which had a higher resistance to INH, also had a mutation in the *inhA* gene. This demonstrated that loss of catalase activity and mutation of *inhA* had a cumulative effect on INH resistance. When a functional *katG* gene was integrated into the genome of WAg405 the INH resistance was reduced from 64 μg/ml to 1 μg/ml. This indicated that most of the resistance had been caused by loss of catalase activity. While the parent INH sensitive strain was virulent for guinea pigs, both the INH-resistant strains were significantly less virulent. Integration of a functional *katG* gene into the most resistant strain restored full virulence. This clearly established that *katG* is a virulence factor for *M. bovis* and that mutation of the *inhA* gene has no effect on virulence.

B3-206 CONSTRUCTION OF A PROMOTER TRAP TO IDENTIFY VIRULENCE GENES IN MYCOBACTERIUM TUBERCULOSIS, Fernanda M. da Silva-Tatley and Mario R.W. Ehlers, Department of Medical Bichemistry, University of Cape Town, Medical School, Observatory 7925, South Africa

Mycobacterium tuberculosis (*M.tb.*) infects one-third of the world's population and is responsible for 3 million deaths annually. Despite intense biological and biochemical research, the pathogenesis of mycobacterial disease remains poorly understood. Progress has been made with the recent development of molecular techniques for mycobacteria. A major goal of current research in mycobacteria is the development of expression systems to study genes required for infection and survival of *M.tb.* within the macrophage. The expression of some *M.tb.*, *M. leprae* and *M. bovis* BCG genes has been investigated in *Escherichia coli* and *Streptomyces lividans*. However, these are heterologous expression systems which do not faithfully represent the mycobacterial molecular machinery, necessary for the organism to respond to host environmental stresses. We have constructed a library of size-restricted (50-600 bp) *M.tb.* genomic DNA in a promoter trap vector, pPTV100. Plasmid pPTV100 is a shuttle vector between mycobacteria and *E. coli* and is based on pMV261 from which the hsp60 promoter has been deleted and a promoterless CAT gene cloned into the *Nhe*I site. *M.tb.* H37Rv transformed with this library is used to infect human monocyte-derived macrophages in culture, and following infection the macrophages are treated with (30 μg/mL) chloramphenicol, which we have shown is mycobactericidal. Only recombinant bacilli containing appropriate promoter elements that are activated intracellularly are expected to survive; these will be recovered, and the promoter elements rescued and used as probes to clone the associated virulence genes.

B3-207 MUTAGENESIS OF MYCOBACTERIA BY RESTRICTION ENYZME MEDIATED INTEGRATION (REMI) OF PLASMID DNA, Raymond Donninger, Fernanda M. da Silva-Tatley and Mario R.W. Ehlers, Department of Medical Biochemistry, University of Cape Town, Medical School, Observatory 7925, South Africa

Mycobacterium tuberculosis (*M.tb.*) is the causative organism of tuberculosis, which worldwide causes 3 million deaths annually. A molecular understanding of the virulence mechanisms of *M.tb.* requires an efficient means of generating genetically tagged random mutants. To date, chemical and insertional mutagenesis strategies in *M.tb.* and *Mycobacterium bovis* BCG have had a poor success rate in generating random mutants. This has been attributed to: (1) the high degree of illegitimate recombination observed in these slow-growing mycobacteria; (2) the fact that mutagenic techniques have relied on homologous recombination events. We are optimising a strategy of illegitimate recombination (REMI), in which pMV261Δ - an autonomously replicating plasmid in *Escherichia coli* containing a kanamycin resistance cassette but no mycobacterial *ori*V - is linearised with *Xba*I, and then electroporated together with active *Xba*I into *Mycobacterium smegmatis* or *M.tb.* H37Rv. Rescue of pMV261Δ and identification of the site of insertion can be achieved by restricting isolated mutant DNA with an enzyme that does not cut the inserted vector, followed by self-ligation and transformation into XL-1 Blue *E. coli*. Kanamycin-resistant REMI transformants will be screened for defects in adherence, internalisation or survival in macrophages.

B3-208 CATALASE REGULATION IN MYCOBACTERIA.

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Strains of mycobacteria deficient in catalase activity are also resistant to isoniazid, a first line mycobacterial antibiotic. Therefore we initiated a study of whether and under what conditions the levels of catalase may vary in various species of mycobacteria. Catalase specific activities were measured under varying conditions of growth or H_2O_2 treatment in *M. smegmatis*, a fast growing, nonpathogenic species, BCG, a slow growing nonpathogenic species and *M. avium*, an intermediately slow growing species which is pathogenic for immunocompromised individuals. The results show that catalase/s are induced in late stationary growth phase in *M. smegmatis*, but not in BCG. In contrast, H_2O_2 treatment causes the induction of catalase activity in BCG, but not in *M. smegmatis*.

In a related study, we found that a multicopy plasmid causing the overproduction of the ribosomal protein S13 from mycobacteria, results in the overproduction of catalase. Strains carrying such genetic constructs are also hypersensitive to isoniazid and have a small colony phenotype. This effect was observed in *M. smegmatis* but not in BCG. We believe overproduction of S13 causes unbalanced growth in *M. smegmatis*, but not in BCG, and this results in a stress response causing the increased production of catalase.

B3-209 INVOLVEMENT OF L7/L12 IN DTH REACTION IN MYCOBACTERIAL INFECTIONS, Yolanta Fishman and Herve Bercovier, Dept. of Clinical microbiology Hadassa med. school The Hebrew University Jerusalem. 91120

Delayed type hypersensitivity (DTH) is one of the earliest manifestations of cellular immunity elicited by mycobacteria and other intracellular pathogens. DTH is the immunological state in which lymphocytes and macrophages are sensitive to components of the pathogen. It is mediated by T-lymphocytes, highly specific and of long duration. This reaction can be studied when fractions of the pathogen are injected into a sensitized host intradermally. As a result of this sensitivity, these fradtions cause a local accumulation and activation of lymphocytes and macrophages, initiating an inflammatory process similar to that characteristic of tuberculosis. Tuberculin or PPD (purified protein derivative) is traditionally used in the case of mycobacteria. PPD is composed of a digest of a mixture of proteins and it does not allow the analysis of specific defined proteins. Few proteins were identified as immunogenic in the DTH reaction. One of these proteins is the ribosomal protein L7/L12. Its activity has been demonstrated recently both in *M. bovis* and *Brucella melitensis* . In this study we present the cloning analysis and expression of the genes encoding L7/L12 from *M. tuberculosis, M. bovis* and *M. avium* . The availability of these three genes and their products enables us to study the involvement of mycobacterial L7/L12 proteins and their specificity in the DTH reaction, to evaluate if the protein is not only immunogenic but can induce DTH reactions as well and to assess a potential protective effect of L7/L12 in mycobacterial infections. Analysis of the genes shows that the homologies between the genes varied between 97.6% and 96.6%. The recombinant proteins were purified to homogeneity and injected to sensitized Guinea pigs. Preliminary results showed that the recombinant L7/L12 protein was immunogenic in the DTH reaction .

B3-210 RECOMBINANT BCG REPORTER STRAINS FOR RAPID ASSESSMENT OF ANTI-MYCOBACTERIAL ACTIVITY *IN VIVO*. Mark J. Hickey, Taraq M. Arain, Ribhi M. Shawar, Devinder. Singh and C. Kendall Stover. PATHOGENESIS CORP. SEATTLE, WA.

Methods for evaluating the antimicrobial activity of compounds on *M. tuberculosis* in the intracellular or *in vivo* environments are extremely arduous involving laborious agar plate colony forming unit (CFU) determinations and long incubations. Recent use of recombinant mycobacterial reporter strains expressing firefly luciferase has led to rapid *in vitro* drug susceptibility testing but the use of mycobacterial reporter strains has not been investigated in macrophages or animals. We constructed a variety of integrative and extrachromosomal firefly luciferase (FF*lux*) reporter vectors with the aim of developing mycobacterial reporter strains for use in intracellular and *in vivo* drug susceptibility studies. A comparison of recombinant BCG reporter strains expressing FF*lux* (rBCG-*lux*) was made in both macrophages and mice allowing the selection of suitable expression vectors for each environment. Five anti-mycobacterial drugs acting by different mechanisms were tested on rBCG-*lux* in infected human THP-1 macrophages and mice. In macrophages, it was possible to observe the inhibitory activity of drugs within five days after infection by directly measuring the reduction of rBCG-*lux* luminescence on infected macrophages. Mice infected with another rBCG-*lux* reporter strain supported easily detectable rBCG-*lux* growth for at least fourteen days as measured by both CFU and luminescence assays (Lux) directly in spleen homogenates. In comparison to untreated control mice rBCG-*lux* dependent luminescence in spleens and livers was substantially reduced by each of five standard drugs within one week of daily treatment initiation. We also compared naive immune competent mice with chemically immunosuppressed mice and BCG-immunized mice for their ability to support the growth of our rBCG-*lux* reporter strain. rBCG-*lux* proliferated to higher levels in immunosuppressed mice beyond three weeks in comparison to normal immunocompetent mice while growth in BCG immunized mice was immediately suppressed, suggesting that the use of mycobacterial reporter strains *in vivo* could enable initial rapid evaluation of immunogens against the mycobacteria. These studies with avirulent rBCG-*lux* reporter strains demonstrate that the use of mycobacterial reporter strains can be extended from *in vitro* applications to intracellular and *in vivo* studies. The use of this technology has the potential to facilitate and accelerate the development of new anti-mycobacterial drugs and vaccines.

B3-211 GENE REPLACEMENT IN MYCOBACTERIA, Jason Hinds, Tanya Parish and Neil G. Stoker, Bacterial Molecular Genetics Unit, Department of Clinical Sciences, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK

The power of molecular genetics is that it can dissect complex biological processes by knocking out individual genes and observing the effects. The production of defined mutants in *M. tuberculosis* by gene replacement has been reported to be much more inefficient than in the fast-growing mycobacterial species, perhaps because of a surprisingly high level of illegitimate recombination. We have been initially studying gene replacement in *M. smegmatis* using the *hisD* gene encoding histidinol dehydrogenase, which catalyses the final step in histidine biosynthesis. We cloned *hisD* into a pUC-derived plasmid carrying the kanamycin-resistance gene, and introduced the hygromycin-resistance gene into *hisD*. Following transformation into *M. smegmatis*, 50 hygromycin-resistant colonies were isolated, of which seven were kanamycin-sensitive. However, all of the colonies grew on minimal medium, indicating that they contained a functional *hisD* gene. Southern blotting showed that in all cases, the plasmid had integrated by illegitimate rather than homologous recombination.

Other groups have reported that gene replacement in *M. smegmatis* is straightforward, and that illegitimate recombination is only high in slow-growing mycobacteria. Our results suggest that illegitimate recombination is also high in fast-growing species. We are now using different strategies including the use of linear DNA to obtain the desired recombinants. In addition, we have cloned the *hisD* gene from *M. tuberculosis* in order to carry out parallel experiments in this species.

B3-212 CLONING OF THE *rpsL* and *rpsG* GENES OF M. SMEGMATIS AND THE USE OF PLASMIDS CARRYING A *xylE* REPORTER GENE TO ANALYZE PROMOTER FUNCTION AND GENE EXPRESSION. Teresa J. Kenney and Gordon Churchward, Department of Microbiology, Emory University, Atlanta GA 30322.

The *rpsL* and *rpsG* genes of *M. smegmatis* were cloned by using non-degenerate oligonucleotides complementary to conserved regions of the *rpsL* gene from 11 different bacteria to amplify a DNA fragment internal to the gene. This fragment was used to screen libraries of chromosomal DNA fragments. The nucleotide sequence of the *rpsL* and *rpsG* genes and upstream regions was determined. The coding regions of the two genes overlapped suggesting that they were arranged as an operon. A combination of primer extension and nuclease protection experiments was carried out to map the 5' end of the *rpsL* mRNA. To identify the promoter of the *rpsL* gene, a new vector plasmid capable of replication in both *E. coli* and *mycobacteria* was constructed that contained a promoterless *xylE* gene. Different DNA fragments from the upstream region of the *rpsL* gene were cloned into the vector to determine which fragment could direct expression of *xylE*. Colonies expressing *xylE* became bright yellow when sprayed with a solution of catechol. The sensitivity of the *xylE* color test was sufficient that single mutant colonies not expressing *xylE* could easily be identified. To determine the role of the upstream region in gene expression, a combination of mutagenic PCR amplification and oligonucleotide-directed mutagenesis was performed to isolate point mutations in the upstream region. The *xylE* reporter system was used to identify and characterize the effects of the mutations.

B3-213 INFLUENCE OF *MYCOBACTERIUM TUBERCULOSIS* CATALASE GENE (*KatG*) EXPRESSION ON THE INTRACELLULAR GROWTH OF TRANSFECTED *MYCOBACTERIUM SMEGMATIS* STRAINS AND NITRIC OXIDE PRODUCTION WITHIN MURINE AND HUMAN MACROPHAGES.

David A.Lammas [1], Nadeem Fazal [1], Pam Drysdale[1], Christine Burt[2], Dinakantha S. Kumararatne[1], and Stewart Cole[3]. Departments of Immunology [1], and Infection[2], University of Birmingham B15 2TT, UK and Unite de Genetique Moleculaire Bacterienne[3] Institute Pasteur, 75724 Paris, Cedex 15, FRANCE.

Strains of *M.smegmatis* with an inactive catalase-peroxidase gene (*KatG*), or into which the *KatG* gene of *Mycobacterium tuberculosis* had been transfected, were used to study the influence of catalase on nitric oxide (NO) production and mycobacterial survival within infected murine and human macrophages. The five strains were observed to grow at similar rates within activated murine or human macrophages over a 3 day infection period irrespective of their catalase phenotype, although initial inhibition of bacterial growth was observed within human macrophages with all strains over the first 24 hours post-infection. High levels of nitrite (40-70nmol/ml) were detected in IFN-γ activated murine but not human cell culture supernatants (<3nmol/ml) but NO_2^- titres produced by infected murine cells did not differ significantly between strains. Possible catalase-mediated resistance to NO killing was also evaluated within cell-free cultures by assessing strain survival against titrations of sodium nitroprusside (SNP), as an *in vitro* generator of NO. SNP however inhibited the growth of all 5 strains of *M.smegmatis* at concentrations of >10mM.

B3-214 IDENTIFICATION OF SPECIFIC MUTATIONS RESPONSIBLE FOR RIFAMPIN AND RIFABUTIN RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* USING A MUTAGENESIS AND EXPRESSION SYSTEM OF THE CLONED *M. TUBERCULOSIS RPOB* GENE, Lincoln P. Miller, Jack T. Crawford, and Thomas M. Shinnick, Division of Bacterial and Mycotic Diseases, CDC, Atlanta, GA 30333

To define the mechanism of rifampin resistance, we cloned and sequenced the *rpoB* gene of *Mycobacterium tuberculosis* and expressed its gene product (the ß subunit of RNA polymerase) in *M. smegmatis* [L. P. Miller, J. T. Crawford, T. M. Shinnick, *Antimicrob. Agents Chemother.* 38, 705 (1994)]. Based on this study, we developed a method to characterize the role of a specific mutation in rifampin and rifabutin resistance in *M. tuberculosis*. The 5.1-kb genomic *Sac*I fragment carrying the *M. tuberculosis* H37Rv *rpoB* gene was cloned into the shuttle plasmid pMV261 to create pLN-2. Using PCR mutagenesis techniques, we introduced a single point mutation into the cloned *rpoB* gene of pLN-2, (designated pMR-1), changing the serine (TCG) codon at position 2430-2 (of the *Sac*I fragment) to leucine (TTG), corresponding to residue 456 of the *M. tuberculosis* ß subunit. This mutation has been noted to occur frequently in clinical strains of rifampin-resistant *M. tuberculosis* and has also been associated with cross-resistance to rifabutin. The desired mutation in pMR-1 was confirmed by nucleotide sequencing, and pMR-1 was electroporated into rifampin-susceptible *M. smegmatis* strain LR222. The MIC of rifampin for both LR222 and LR222(pLN-2) was 25 µg/ml. In contrast, LR222(pMR-1) was more resistant to rifampin (MIC=200 µg/ml). The MIC of rifabutin for both LR222 and LR222(pLN-2) was 2.5 µg/ml, whereas LR222(pMR-1) was resistant to rifabutin (MIC=25 µg/ml). We are using additional mutants of pLN-2 to characterize different *M. tuberculosis rpoB* mutations implicated in rifampin resistance. This study will allow us to discriminate between the *rpoB* mutations, which are solely responsible for rifampin resistance, and the subset of *rpoB* mutations, which is responsible for rifampin and rifabutin resistance. In addition, our method is applicable to the study of other cloned drug-resistance genes of *M. tuberculosis* because it can be used to distinguish mutations responsible for drug resistance from differences due to sequence polymorphisms.

B3-215 CLONING, OVEREXPRESSION AND ENZYMOLOGY OF THE DNA POLYMERASE I FROM *MYCOBACTERIUM TUBERCULOSIS*, Valerie Mizrahi[1], Douglas R. Smith[2], and Pieter Huberts[1]. [1]Molecular Biology Unit, South African Institute for Medical Research and Department of Haematology, University of the Witwatersrand, Johannesburg, South Africa; [2]Genome Sequencing Center, Collaborative Research Incorporated, 1365 Main Street, Waltham, MA. 02159, USA.

The *polA* gene encoding the DNA polymerase I (PolI) from *M. tuberculosis* was cloned using a probe generated by consensus sequence PCR amplification of genomic DNA (Mizrahi, V., Huberts, P., Dawes, S.S. and Dudding, L.R. (1993) *Gene* **136**, 287-290). The G+C content of the structural gene is 67% and it encodes a polypeptide of 904 amino acids that shares 89% amino acid identity with the 911-amino acid homologue from *M. leprae*. The *M. leprae polA* gene is flanked by similar but not identical RLEP elements arranged in a convergent inverted repeat orientation that are spaced 21 and 1 base pairs, respectively, from the start and stop codons of the gene and thus, structurally resembles a transposable element. The *M. tuberculosis* PolI was overexpressed in *E. coli* and was purified to homogeneity and enzymatically characterized. The enzyme catalyzed DNA polymerization with a significantly lower k_{cat} for homopolymer synthesis than its *E. coli* counterpart, but it lacked a proofreading 3'-5' exonuclease activity, in accordance with the absence of the conserved ExoI-III subdomains ordinarily required for this function. Although the 5'-3' exonuclease activity was undetectable in the absence of DNA synthesis, this reaction was significantly activated when the scissile 5'-terminus is located in advance of an extending primer terminus.

B3-216 IMMUNOPOTENTIATING EFFECTS OF TUMOR NECROSIS FACTOR (TNF) IN MYCOBACTERIAL DISEASES, Surjit R. Moolamalla and Machireddy Narsa Reddy, University College of Science, Osmania University, Hyderabad - 500007, India.

Protection against mycobacterial infections can be inferred by the administration of cytokines which potentiate the immune responses. Of all the cytokines produced in response to mycobacteria TNF plays central role by activating immune mechanisms that enhance mycobacterial elimination.

Peripheral blood mononuclear cells from the tuberculosis and leprosy patients were challenged with sonicated M. Leprae, sonicated BCG, 65 KD (M, tuberculosis) and 16 KD (M, Leprae) recombinant proteins and lipo arabinomannan (M Leprae) along with and without recombinant TNF added to the culture medium at 5 IU, 10 IU, 15 IU, 20 IU concentrations. The effect of TNF on antigen presentation (KLH) MHC class II expression, production of TNF , IL-1 and Oxygen radicals (O2- and H202) was studied in vitro. TNF at 5 IU, 10 IU enhanced the KLH presentation MHC class II expression and production of TNF and IL-1 to all the antigens in Tuberculosis and Tuberculoid leprosy patients whereas at 15 IU and 20 IU concentrations the above said immune functions diminished may be due to the cytotoxic effect of this cytokine. Oxygen radicals production enhanced to all the concentrations of TNF. In lepromatous leprosy patients TNF at all concentrations did not show any effect on all the immune functions in response to all the antigens.

These results indicate immunopotentiating and proinflammatory effect of TNF which facilitate the elimination of mycobacteria. The implication of these results in immunotherapy of Tuberculosis and leprosy will be discussed.

B3-217 EXPRESSION OF HETEROLOGOUS GENES IN NOVEL MYCOBACTERIAL VECTORS, Peadar O'Gaora, Chris Hayward, Jelle Thole and Douglas Young, Dept. Medical Microbiology, St. Mary's Hospital Medical School, Norfolk Place, London, W2 1PG, U.K.

The recent development of vectors which can replicate autonomously or integrate in the chromosome has been a significant step in the field of mycobacterial research. To date however, reports of the use of selective agents other than kanamycin have been sparse. We previously described a vector (p16RI) based on hygromycin resistance which was selectable in a wide range of mycobacterial species. In this report, we describe an expanded range of vectors which use this selection and their use in heterologous gene expression in mycobacterial hosts. The new vectors include the incorporation of a polylinker in p16RI and the introduction of various promoters. In addition, plasmids which allow targetted localization of antigens have been constructed. We have expressed, in BCG and in the fast growing species, *M.smegmatis* and *M. vaccae*, the B subunit of the *Escherichia coli* heat-labile toxin (LT-B) under the control of the hsp60 promoter and as a secreted lipoprotein fused to the N-terminus of the 19kDa antigen. The dust mite antigen, Der-P2, has been expressed from both the hsp60 and superoxide dismutase (SOD) promoters. Comparison of the level of expression will be made with that of the same genes integrated in the chromosome. We are currently using these recombinant mycobacteria to investigate various aspects of host immune responses in animal models of allergy and oral tolerance.

B3-218 CONDITIONAL VECTORS FOR USE IN MYCOBACTERIA Tanya Parish and Neil G. Stoker. Bacterial Molecular Genetics Unit, Department of Clinical Sciences, London School of Hygiene & Tropical Medicine, London, England, WC1E 7HT.

Mutagenesis of *M. tuberculosis* by transposons or by gene replacement methods are vital tools which are needed for studying this major pathogen. The efficiency of these procedures can be improved by the use of conditional vectors which are unable to replicate in the host organism under certain conditions. The classical approach is to isolate plasmids or phages with a temperature-sensitive replication phenotype. A temperature-sensitive plasmid has been created which has been used successfully as a transposon delivery vector in *M. smegmatis*. However, the narrow temperature range at which *M. tuberculosis* grows means that the plasmid is not suitable in this species.

We have been using an alternative approach to develop a conditional plasmid vector. The acetamidase of *M. smegmatis* is highly induced by the presence of acetamide. We have evaluated several regions of this gene and the upstream region for promoter activity using a chloramphenicol acetyl transferase reporter gene and have identified regions giving inducible and constitutive expression. Several constructs have been made linking the promoter regions of the acetamidase to the replication regions of the pAL5000 plasmid in order to see if the high levels of transcription from the acetamidase promoter under induced conditions will interfere with or block plasmid replication. These constructs are currently being evaluated for their replication in the presence and absence of acetamide.

B3-219 CONSTRUCTION OF PLASMID VECTORS TO PRODUCE ANTISENSE RNA IN MYCOBACTERIA Bonnie B. Plikaytis and Thomas M. Shinnick, Division of Bacterial and Mycotic Diseases, CDC, Atlanta, GA 30333

A key step in the analysis of a cloned gene is to elucidate its biologic function in the native organism. Classically, this has been done by constructing a "knock-out" mutant by site-directed mutagenesis of the cloned gene followed by replacement of the wild-type gene with the mutated gene by homologous recombination. Unfortunately, even with the recent advances in the study of *Mycobacterium tuberculosis*, reliable homologous recombination has not yet been achieved. An alternative approach to gene replacement for producing a knock-out mutant is to use antisense RNA to inhibit mRNA translation. This approach has been successfully utilized in bacteria, plants, and eukaryotic organisms. The first step in this approach is to produce RNA that is complementary to the mRNA. Theoretically, this antisense RNA anneals to the mRNA and inhibits its translation; thereby blocking gene expression. Two plasmid vectors were constructed to produce antisense RNA in mycobacteria. Plasmids pBP1 and pBP2 utilize the *cpn*60-2 (*M. tuberculosis* 65-kD antigen gene) promoter to transcribe antisense RNA from a DNA sequence inserted into a *Bam*HI site downstream of the promoter sequence. Plasmid pBP1 contains an *ori*E and *ori*M and the kanamycin resistance gene, which allows it to be shuttled between *E. coli* and mycobacteria. Plasmid pBP2 contains *ori*E to allow replication in *E. coli* and the L1 integrase and attachment site to allow integration into the mycobacterial genome. These vectors should be useful tools in blocking gene expression, which could aid in the study of the pathogenicity of mycobacteria.

B3-220 IDENTIFICATION OF *Mycobacteriun. tuberculoisis* DNA SEQUENCES ENCODING EXPORTED PROTEINS, USING *PhoA* GENE FUSIONS, Denis Portnoï, Eng-Mong Lim, François-Xavier Berthet, Juliano Timm, and Brigitte Gicquel, Unité de Génétique Mycobactérienne, CNRS URA 1300, Institut Pasteur, 75015 Paris, France.

The activity of bacterial alkaline phosphatase (PhoA) is dependent on it being exported across the plasma membrane. A plasmid vector (pJEM11) allowing fusions between *phoA* and gene encoding exported proteins was constructed to study protein export in *Mycobacteria*. Introduction of the *M. fortuitum* β lactamase gene (*blaF* *) into this vector led to the production in *M. smegmatis* of protein fusions with PhoA activity. A genomic library from *M. tuberculosis* was constructed in pJEM11 and screened in *M. smegmatis* for clones with PhoA activity. Sequences of the *M. tuberculosis* inserts directing PhoA export were determined. They include part of the already known exported 19 kDa lipoprotein, a sequence with similarities with the exported 28 kDa antigen from *M. leprae*, a sequence encoding a protein sharing conserved amino acid motifs with stearoyl-acyl-carrier-protein desaturases, and unknown sequences. This approach thus appears to identify sequences directing protein export, and we expect that more extensive screening of such libraries will lead to a better understanding of protein export in *M. tuberculosis*.

B3-221 IDENTIFICATION OF THE MOLECULAR TARGET FOR ISONIAZID IN *MYCOBACTERIUM TUBERCULOSIS* .

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The majority of the multi-drug-resistant *tuberculosis* strains are resistant to isoniazid (INH), one of the most effective antituberculosis drugs. Determining the molecular mechanism of action of isoniazid became crucial both for understanding resistance mechanisms arising in mycobacteria and for designing more potent anti-mycobacterial drugs in order to develop novel approaches for controlling tuberculosis. Genetic studies have recently identified a mycobacterial gene, *inhA*, which encodes a common target for isoniazid and ethionamide action. We have demonstrated that the *M. tuberculosis* InhA protein catalyzes an NADH-dependent 2-*trans*-enoyl-ACP reduction, essential for fatty acid elongation, and most likely involved in the mycobacteria-specific mycolic acid pathway. Kinetic analyses of the wild type and the S94A mutant, known to confer resistance to both INH and ethionamide, suggest that the mechanism of resistance resides in a decreased affinity (5-10 fold) of the enzyme for NADH. The parameters of inhibition of InhA proteins by INH imply that isoniazid itself is a pro-drug, which is converted to an active form. Our data allow us to propose a model of action of isoniazide on its target, the InhA protein.

B3-222 **The Stationary Phase of *M. tuberculosis*,** Anjali Seth, Nancianne Knipfer & Thomas E. Shrader, Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461

Reactivation tuberculosis is expected to require the exit of mycobacterial cells from a dormant "stationary" phase to an actively dividing phase. Experiments designed to increase our understanding of the prolonged survival of *M. tuberculosis* cells in stationary phase are currently underway in our laboratory.

Identification of promoters induced during entry and exit from the stationary phase of *M. tuberculosis*. A series of regulated promoter element cloning vectors allowing selection for ON and OFF states are being used to identify promoter sequences differentially regulated during the stationary phase. Regulated promoter elements are selected based on concomitant expression of the genes encoding the orotidine decarboxylase enzyme (toxic when expressed in growing cells in the presence of 5'-FOA) and an antibiotic resistance marker arranged in an operon. These vectors allow identification of those proteins specifically synthesized during entry of the stationary phase of *M. tuberculosis* as well as the enzymes that are synthesized during reactivation.

Identification of gene products essential for survival during the stationary phase of *M. tuberculosis*. Transposon mutagenesis is being employed to generate and identify chromosomal insertions which result in a lack of stationary phase survival. These studies involve the generation of ~10,000 independent chromosomal insertions into the mycobacterial chromosome followed by screening for those insertions resulting in rapid cell death during prolonged growth under conditions of limited culture aeration. This screen is expected to identify genes required for onset of a stable stationary phase, survival during stationary phase, and the programmed exit from the stationary phase to a growing and dividing phase.

Characterization of the role of proteolysis in cell survival and the maintenance of amino acid pools during prolonged periods of stationary phase. Protein degradation forms an essential part of the protective response employed by both bacterial and eukaryotic cells during stationary phase survival. We have demonstrated that the Lon protease is essential in *M. smegmatis*. Genetic schemes are described which are designed to characterize the essential role of the Lon protease in mycobacterial physiology and to identify essential proteases from *M. tuberculosis*.

B3-223 MYCOBACTERIAL TRANSCRIPTION APPARATUS,

Issar Smith, Mima Predich, Laurence Doukhan and Gopalan Nair, Public Health Institute, New York, NY 10016

To understand how expression of mycobacterial virulence genes is controlled in the macrophage, we are initially studying the transcriptional machinery of pathogenic and non-pathogenic members of this genus. We purified RNA polymerase holoenzyme from *M. smegmatis* and showed that it can transcribe homologous and heterologous genes. Promoter-specific transcription ability was eliminated when two proteins, immunoreactive to a sigma factor specific monoclonal antibody, were removed from the core polymerase. This activity was restored when the major vegetative sigma factor (sigma A) from *Bacillus subtilis* was added to the core enzyme. These results suggest that the removable proteins are sigma factors. We cloned two genes, *mysA* and *mysB*, coding for sigma factors of the sigma 70 class, from both *M. smegmatis* and *M. tuberculosis*. *mysA* is thought to encode the essential housekeeping sigma factor as it shows very high similarity to the major sigma factor of *Streptomyces*, HrdB. Genetic inactivation experiments were consistent with this conclusion which further showed that *mysB* seems dispensible for growth. Protein microsequencing of internal peptides from the two sigma proteins of the *M. smegmatis* holoenzyme(s) showed that they are encoded by *mysA* and *mysB*, indicating that both genes are expressed during exponential growth. PCR and sequence analysis indicated that the two sigma genes are closely linked, about 3.5 Kb apart, and the same two genes and chromosomal organization were found in all mycobacteria so far analyzed (*M. smegmatis, M. tuberculosis, M.leprae, M.bovis BCG*, and *M. avium*). Immediately downstream of *mysB* in both *M.smegmatis* and MTB was found a gene encoding a homolog of the *Corynebacterium diptheriae* toxin gene repressor, DtxR (this region in M. leprae has not yet been sequenced). Thus the chromosomal organization of this region in mycobacteria is *mysA, mysB, dtxR*, on a DNA segment of approximately 7 Kb (work supported by NIH grant GM 19693).

B3-224 CLONING OF AN UNUSUAL *RECA* ACTIVITY IN *MYCOBACTERIUM TUBERCULOSIS*. Lafras Steyn, Karen Shires, Shamila Nair and Harold Zappe, Department of Medical Microbiology, University of Cape Town, Observatory, Cape Town, 7925, South Africa

We have previously reported the cloning of a RecA-like activity from *Mycobacterium tuberculosis* (Nair & Steyn, *J Gen Microbiol*, 137, 2409-2414, 1991). This activity was able to complement *recA E. coli* cells for EMS and UV resistance and for homologous recombination. Sequence analysis of the cloned DNA demonstrates an ORF encoding a protein of 463 amino acids. The ORF463 is truncated at the 5'-end and in the same orientation, but not in frame with, the β-galactoside gene of pUC19. Plasmids containing ORF463 are unstable and undergo spontaneous rearrangements. Interestingly only 20% of *E. coli* cells transformed with pUC19 containing the ORF463 are UV resistant; the ORF463 could be expressed due to genetic decoding or due to rearrangements of the plasmid. At present we are sequencing several rearranged plasmids to determine if ORF463 is affected by the rearrangements. In addition we are using PCR to clone the entire gene.

We have also made a Maltose Binding Protein - ORF463 gene fusion and are using the protein cleaved from the MBP to generate monoclonal antibodies for use in studies on the native protein.

B3-225 PLASMID INCOMPATIBILITY IN MYCOBACTERIA Pelle Stolt and Neil G. Stoker
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Genetic manipulation of mycobacteria has been possible for several years, using electroporation and *E. coli*-mycobacteria plasmid shuttle vectors. Even so, little is known about plasmid replication and stability in these organisms. Efficient shuttle vectors have largely been constructed by trial and error, trying to define the smallest piece of a plasmid that will suffice for replication in mycobacteria.

We are investigating the determinants of plasmid stability, with an aim to defining partitioning-mediating regions, using *Mycobacterium smegmatis*. We are studying the propagation of pairs of plasmid derivatives all based on the *M. fortuitum* plasmid PAL5000. Since they only differ in the genetic marker carried, these plasmids should be incompatible and only be retained under selection pressure for both resistances. The degree of incompatibility was determined by plating out under one or the other selective pressure and scoring for degree of plasmid loss. Different regions are being deleted or inserted and the effects of these manipulations on plasmid incompatibility are being assessed.

B3-226 CLONING OF A COMPLETE TWO-COMPONENT REGULATORY SYSTEM OF *MYCOBACTERIUM BOVIS* BCG, Philip Supply and Camille Locht, Laboratoire de Microbiologie Génétique et Moléculaire, Institut Pasteur de Lille, 1, Rue du Professeur Calmette, F-59019 Lille, France.

Despite the importance of mycobacteria, including *Mycobacterium tuberculosis*, as human pathogens, very little is known about their virulence factors and their genetic regulation in response to environmental stimuli. Studies with numerous other bacterial pathogens, including intracellular microorganisms, have shown that in most cases the production of virulence factors is co-ordinately regulated at the transcriptional level. This regulation generally occurs through a two-component system in which one component senses changes in the environment and transmits this information via phosphorylation to the second component. Once phosphorylated, the second component functions as a transcriptional transactivator. The N-terminal domains of the transactivators contain well conserved sequences. On the basis of this homology, a 0.26 kb gene fragment encoding the N-terminal domain of an unknown response regulator from *M. tuberculosis* had previously been isolated. Using the polymerase chain reaction technique, we amplified the corresponding DNA fragment from *Mycobacterium bovis* BCG. This DNA fragment was then used to isolate a 3.3 kb fragment from a partial BCG DNA library cloned into pBluescript. Sequence analysis indicates that it contains the complete gene encoding a sensor protein followed by the gene coding for a transcriptional activator. Particularly high sequence similarities are found between this sensor and phosphate sensors from other microorganisms. In addition, the C-terminal domain of the activator also shows high sequence similarities with activators of phosphate regulons. A highly homologous system is also present in *Mycobacterium leprae*. However, interestingly, Southern blot analyses suggest that it is not present in the non-pathogenic *Mycobacterium smegmatis*. The isolated genes can now be used to identify target genes of this regulon.

B3-227 AN EFFLUX PUMP IN Mycobacteria smegmatis CONFERS LOW-LEVEL FLUOROQUINOLONE RESISTANCE. H.Takiff[1], M.Cimino[1], M.Musso[1], M.Delgado[1], T.Weisbrod[2], R.Martinez[1], A. Telenti[3], B.R.Bloom,[2] and W.R.Jacobs,Jr.[2]. IVIC, Caracas, Venezuela[1], HHMI, Albert Einstein Coll. of Med, Bronx, NY[2], and Inst. for Med. Micro., Berne, Switzerland[3].

The fluoroquinolones (FQ) have become accepted in the therapy of multidrug resistant strains of M.tuberculosis, but FQ resistant strains have begun to appear. High level FQ resistance is generally associated with mutations in gyrA, the gene encoding the A subunit of the DNA gyrase, but there is evidence in other species that resistance develops as a multistep process. To identify other genes involved in FQ resistance in mycobacteria, we isolated a high-level ciprofloxacin resistant (Cip^r) M.smegmatis mutant (mc^2551) by successive selections on increasing drug concentrations, and then electroporated a shuttle plasmid genomic library made from this strain into Cip^s M.smegmatis. Clones conferring Cip resistance contained a gene encoding a protein of the Major Facilitator family of proton antiporter efflux pumps, which was named mfr, for mycobacterial FQ resistance. Mfr is most homologous to QacA from S.aureus, but is also homologous to ActII, TcmA, and Mmr--polyketide secretion pumps from Streptomyces. Like the more distantly related pump NorA, Mfr confers more resistance to the hydrophilic FQs such as Cip, than to the hydrophobic FQs such as sparfloxacin. The original, highly resistant strain mc^2551 also contains a gyrA mutation.

B3-228 TRANSCRIPTIONAL ANALYSIS AND REGULATION OF THE *MYCOBACTERIUM TUBERCULOSIS* 16S rRNA-ENCODING GENE, Jaya Sivaswami Tyagi and Anita Verma, Department of Biotechnology, All India Institute of Medical Sciences, New Delhi-110029, India.

rRNA synthesis constitutes a major fraction of the RNA synthesis in *Mycobacterium tuberculosis*. The genome of *M. tuberculosis* carries only one rRNA operon, thus this organism is a good candidate for studying the regulation of rRNA synthesis. A functional analysis of *M. tuberculosis* 16S ribosomal RNA (rRNA) transcription and processing was undertaken. RNA:DNA hybridizations indicated that the maximum transcriptional activity of rRNA-encoding genes (rDNA) corresponded to the earliest period of exponential growth. Transcription start points (*tsp*) were mapped by primer extension analysis of RNA from *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra. Identical hybridization kinetics and primer extension signals indicated similarity in rDNA transcription and processing in laboratory grown cultures. The precursor transcripts are processed into mature 16S rRNA through a pathway that includes recognition of RNA secondary structure by ribonuclease III in the stem structure surrounding the 16S rRNA indicating that at least this RNA processing step is conserved in mycobacteria and *E. coli*. *M. tuberculosis* DNA sequences located upstream of the 16S rRNA-coding region were isolated by inverse PCR. The putative *rrn* promoter region from H37Rv was cloned upstream of the promoterless chloramphenicol acetyltransferase (CAT)-encoding gene in a shuttle vector pSD7 to generate a promoter-fusion construct. The *M. smegmatis* transformant exhibited a CAT activity of 16669 nmol/min/mg of protein suggesting that the 16S promoter was of exceptionally high strength. Two *tsp* utilized in *M. tuberculosis* were also employed in *M. smegmatis*. Modulation in *rrn* promoter activity in response to changes in the nutritional environment will be discussed.

B3-229 GENE ORGANIZATION OF THIOREDOXIN AND THIOREDOXIN REDUCTASE IN MYCOBACTERIA, Brigitte Wieles, Dick van Soolingen, Rienk Offringa, Tom Ottenhoff and Jelle Thole, Department of Immunohematology and Blood Bank, University Hospital Leiden, The Netherlands.

Thioredoxin (TRX), thioredoxin reductase (TRX-R) and NADPH form an integrated system providing electrons to a wide variety of different metabolic processes in prokaryotic and eukaryotic cells. In bacteria TRX and TRX-R proteins thusfar were found to be encoded by separate genes. In this study we report the genomic organisation of TRX-R and TRX in mycobacteria and show that at least three modes of organisation of TRX-R and TRX genes can exist within a single bacterial genus. The majority of mycobacterial strains tested were shown to have the genes coding for TRX-R and TRX on seperate locations on the genome. Interestingly, in all pathogenic *M.tuberculosis* complex mycobacteria both genes are found on the same locus overlapping in one nucleotide. A unique finding was that in contrast to all other mycobacterial strains, in *Mycobacterium leprae*, TRX-R and TRX are encoded by a single gene. The N-terminal part of the protein corresponds to TRX-R and the C-terminal part to TRX. A corresponding single protein product of approximately 49 kDa was detected in cell extracts of *M.leprae*. These findings demonstrate the highly unusual phenomenon of a single gene coding for both the substrate (thioredoxin) and the enzyme (thioredoxin reductase) that seems to be unique to *M.leprae*.

B3-230 IDENTIFICATION OF GENES ENCODING EXPORTED PROTEINS OF *MYCOBACTERIUM TUBERCULOSIS* USING ALKALINE PHOSPHATASE GENE FUSIONS AS A REPORTER SYSTEM. Michael A. Wilson and Gary K. Schoolnik. Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305.

Genetic fusions with the *Escherichia coli* alkaline phosphatase gene (*phoA*) have proven useful in a variety of bacterial systems for identifying and characterizing proteins, or domains of proteins, that are exported beyond the cytoplasmic membrane. We have adapted this technology as a means of screening for *M. tuberculosis* genes that encode exported proteins. Random fragments of *M. tuberculosis* genomic DNA were cloned in front of a "leaderless" phoA gene which is carried on a mycobacterial shuttle vector. *E. coli* and *M. smegmatis* serve as surrogate hosts for expression of the *M. tuberculosis-phoA* fusions. Information contained within the *M. tuberculosis* portion of the fusion protein, if recognized by the host cell machinery, can direct the export of the polypeptide and allow alkaline phosphatase to become enzymatically active. Those clones that have increased alkaline phosphatase activity are readily detected as blue colonies on agar containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). To date, we have identified a number of *M. tuberculosis* DNA fragments that allow alkaline phosphatase to become enzymatically active when the plasmid is present in *M. smegmatis*. Another set of DNA fragments confers a similar activity when expressed in *E. coli*. We are currently analyzing these sets of fragments to identify the sequences involved. This strategy should prove useful for identifying some of the surface-exposed and secreted proteins of *M. tuberculosis*. It should also increase our understanding of the determinants of protein export in mycobacteria.

Host Resistance Mechanisms; Mechanisms of Pathogenesis

B3-300 MOLECULAR AND CELLULAR STUDIES OF THE INTRACELLULAR KILLING OF *MYCOBACTERIUM BOVIS* BY MACROPHAGES FROM GENETICALLY RESISTANT CATTLE, L. Garry Adams, Jianwei Feng, Robert Barthel, Tariq Qureshi, Jorge Piedrahita and Joe W. Templeton, Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4467

Natural (genetic) disease resistance is the inherent capacity of an animal to resist disease without prior exposure or immunization. Cattle naturally resistant to *Brucella abortus* were bred and progeny from five generations of families were used to study the heritability of this trait. Back-crossed calves were phenotyped by challenge with *B. abortus* and the results from genetic analyses indicated that at least two genes control this trait. Macrophages (Mø) from resistant cattle restricted the *in vitro* growth rate of *Mycobacterium bovis, B. abortus* and *Salmonella dublin* significantly and was correlated 83% with *in vivo* resistance. Application of differential display reverse transcriptase PCR (DD RT-PCR) to Mø from either resistant or susceptible cattle infected or non-infected with *M. bovis* or *B. abortus* identified 12 differentially expressed mRNAs which are being characterized for their potential role in controlling the intracellular growth of *M. bovis*. Quantitative expression of TNFα, TNFαR, GM-CSF, TGFβ3, IL-1, IL-3, IL-4, IL-8r, IL-10, IL-12, Nramp, iNOS and selected differentially expressed genes detected by DD RT-PCR is being determined by reverse transcription-T7 RNA dependent amplification (RT-TRDA) in Mø from either resistant or susceptible cattle infected or non-infected with *M. bovis* or *B. abortus*. DNA and cDNAs isolated from cattle having resistance or susceptibility to *B. abortus* were used for genetic analysis with probes linked to resistance to murine tuberculosis (MuMu 1) and human tuberculosis and leprosy (HSA 2q 24,25). Because the major candidate gene, *Bcg* (*Nramp*) responsible for resistance in mice and man is located on MuMu 1 and HSA 2q 24,25 respectively, and the bovine homolog is located on Chromosome 2, efforts were focused on *Nramp* as a major candidate gene. Using RT-PCR amplification of mouse and human homologs of *Nramp*, the bovine *Nramp* gene was identified, sequenced, mapped to bovine Chromosome 2, was expressed exclusively in RES cells, and was demonstrated to be associated with resistance to bovine brucellosis.

B3-301 CULTURE-ACTIVATED MURINE IMMUNE LYMPHOID CELLS ARE NOT SUBJECT TO THE ISOGENEIC BARRIER TO ADOPTIVE IMMUNIZATION AGAINST *MYCOBACTERIUM TUBERCULOSIS*, Ronald A. Barry, Immunology Research 151-R, Department of Veterans Affairs Medical Center, Portland, OR 97201.

Adoptive transfer studies in experimental models of murine tuberculosis suggest that both $CD4^+$ and $CD8^+$ T-lymphocytes function in protection, and that $CD4^+$ T-lymphocytes appear to be the dominant T-cell phenotype involved in the expression of specific immunity. Unfortunately, this expression of adoptively-transferred antimycobacterial immunity is critically dependent on rendering recipient animals immunocompromised by means of sublethal irradiation, chemotherapy, aging, or thymectomy, irradiation and bone marrow reconstitution. These findings suggest that this "isogeneic" barrier to adoptive immunity is mediated by a host-derived T-cell dependent mechanism. In an attempt to overcome this isogeneic barrier to adoptive antimycobacterial immunity, immune splenic lymphoid cells obtained from *M. tuberculosis*-infected C57BL/6 mice were polyclonally activated *in vitro* (with the T-cell mitogen concanavalin A) prior to transfer into irradiated (500 Rads) or non-irradiated normal syngeneic mice. These recipient mice, as well as irradiated and non-irradiated murine recipients of directly transferred (non-cultured) immune splenic lymphoid cells, were simultaneously challenged with *M. tuberculosis* and adoptive immunity determined by assaying relative numbers of bacteria in spleen, liver and lung tissues of recipient mice relative to normal control mice challenged with *M. tuberculosis* only. Results of these studies confirmed that the expression of adoptive immunity mediated by non-cultured immune splenic lymphoid cells was dependent on sublethal irradiation of the murine recipients. In contrast, the culture-activated immune splenic lymphoid cells mediated the expression of adoptively-transferred antimycobacterial immunity in both the irradiated and non-irradiated murine recipients. In addition, this expression of adoptive immunity in the non-irradiated murine recipients was completely eliminated by treatment of the culture-activated immune lymphoid cells with antibody to the Thy 1 (T-cell) antigen and complement. These data suggest that the culture-activation of immune splenic lymphoid cells results in a cellular differentiation event which circumvents this isogeneic barrier to adoptively transferred antimycobacterial immunity.

B3-302 MYCOTIN: A LECTIN INVOLVED IN THE ADHERENCE OF MYCOBACTERIA TO MACROPHAGES, Basu, J. Goswami, S. Sarkar, S. Kundu, M. and Chakrabarti, P., Department of Chemistry, Bose Institute, 93/1 APC Road, Calcutta-700009. India

Pathogenic Mycobacteria colonize host macrophages. One of the steps which merits consideration as a target for developing new approaches for combating Mycobacteria-caused diseases, is the primary step of attachment of the bacteria to the host cell surface. Interest in this approach was enhanced by our finding that the fast growing Mycobacteria, M. smegmatis, secretes an extracellular lectin, mycotin. (Kundu, Basu & Chakrabarti, FEBS Letter, 1989, 156, 207). Although lectins have been implicated in the attachment of several bacterial species to macrophages our studies demonstrate for the first time, the novel involvement of lectin-like proteins on the cell surface of Mycobacteria and identify lectin-sugar interactions as a means of attachment of pathogenic Mycobacteria M. tuberculosis, M. avium, M. kansasii and M. lepre to macrophages. Western blots confirmed the presence of two mycotin-related proteins of M_r 67000 and 35000 in M. avium and M. tuberculosis respectively. Adherence of Mycobacteria to macrophage was significantly inhibited by anti-mycotin antibody or the mycotin-specific sugar, mannan suggesting that mycotin or related-proteins play a potential role in mycobacterial adherence to macrophage. These observations suggest that prevention of the interaction of mycotin-related molecules on the surfaces of Mycobacteria with mannose-specific receptors on macrophages, offers an important approach for blocking attachment of pathogenic Mycobacteria to macrophages, thereby preventing infection.

B3-303 GAMMA DELTA T CELL EXPANSION IN *M.TUBERCULOSIS* INFECTED CULTURES OF HUMAN PERIPHERAL BLOOD CELLS IS POSITIVELY CORRELATED WITH MONOCYTE DEATH Elizabeth A. Bearer, Richard. S. Kornbluth, Department of Medicine, University of California-San Diego, San Diego, CA 92093

In vitro cultures of human peripheral blood cells from TB naive and sensitized donors have been used to investigate relationships between *M. tuberculosis* and mononuclear cells. In the first 24 hours of culture, many monocytes apoptosed in the absence of TB infection. Infection of monocytes at either low (<1 TB: monocyte) or high innoculum (1-4 TB: monocyte) suppressed apoptosis. About the third day of culture, a high TB innoculum resulted in the death of monocytes which was due to an effect of TB rather than lymphocyte cytotoxicity. In cultures of TB naive donor cells at day seven, CD4+ or CD8+ T cells were not expanded. In cultures of PPD+ donor cells, CD4+ T cells proliferated with either low or high TB innoculum but CD8+ T cells did not respond to either innoculum. When cells from PPD+ donors were incubated with a high TB innoculum, gamma delta T cells proliferated. Gamma delta cell proliferation did not occur with a low innoculum. These observations suggest that monocyte death may be important for gamma delta T cell proliferation.

B3-304 NITRIC OXIDE IS NOT INDUCED IN STIMULATED GUINEA PIG MACROPHAGES WITH OR WITHOUT BCG VACCINATION. Sheldon T. Brown[1], Gloria Almeida[2], Howard Doo[2], Ian Brett[1], Suhui He[2], John L. Ho[2]. [1]Bronx VAMC, [1]Mt. Sinai School of Medicine, and [2]Cornell University School of Medicine, NYC, NY.

The absence of inducible nitric oxide synthase (iNOS) activity in human macrophages (MΦ), but the ready detection of high levels in murine MΦ, may explain the relative susceptibility of humans to infection with *M. tuberculosis*. We compared the capacity to induce iNOS activity in guinea pig (GP) alveolar MΦ (ALM), GP thioglycolate elicited peritoneal lavage MΦ (PEM), and a murine MΦ cell line (RAW 264.7). MΦ were plated at 6 x 10^5 cells/cm^2 in microtiter plates and incubated in RPMI 1640 + 10% FCS + lipopolysaccharide @ 2.5 μg/ml (LPS), recombinant human (rHu) or murine (rMU) IFN-γ @ 1000 U/ml, or LPS + rHu or rMu IFN-γ. Supernatents were harvested and assayed for nitrite or for reduction of ferric cytochrome C. Following induction by single or combinations of stimuli, GP PEM and ALM produced no more nitrite than media control after stimulation for 24, 48, and 72 hours. RAW cells produced large quantities of nitrite after stimulation with LPS and/or rMu IFN-γ, but iNOS activity was not affected by rHu IFN-γ. Activation of MΦ, shown by upregulation of superoxide production, was seen from GP MΦ treated with CM but not with recombinant IFN-γ's. After GP were vaccinated or vaccinated/boosted with BCG no nitrite was produced by PEM incubated in media or with LPS despite a vigorous respiratory burst. These data support the hypothesis that stimulated GP MΦ, like human MΦ, lack detectable iNOS activity using methods adequate for inducing murine MΦ iNOS activity. GP may therefore be a more suitable model than mice for studies of host responses to mycobacterial infections.

B3-305 IRON LIMITED GROWTH AND IRON REGULATED PROTEINS OF *Mycobacterium tuberculosis*. Kathleen M. Calder and Marcus A. Horwitz, Department of Medicine, School of Medicine, University of California, Los Angeles, CA 90024

Iron, an essential nutrient of *M. tuberculosis*, plays a central role in the interaction of this pathogen with the host. To learn more about iron acquisition and utilization by *M. tuberculosis*, we studied the effects of a wide range of iron concentrations on bacterial growth and protein expression. Growth of *M. tuberculosis* was optimal at 1 μM iron and above, but decreased at ≤0.5 μM iron. Growth was negligible at ≤ 0.2 μM iron.

To identify iron-regulated proteins (IRPs) of *M. tuberculosis*, we harvested and analyzed growing bacterial cells from cultures containing a limiting amount of iron (0.5μM) or a nonlimiting amount of iron (18 μM). Seven IRPs were identified on analysis by polyacrylamide gel electrophoresis. Expression of three of these proteins (IRP 15, 24, and 29, designated by molecular mass in kilodaltons) was increased during growth at the high iron concentration. Conversely, expression of four proteins (IRP 10, 13, 23, and 28) was increased during growth at the low iron concentration. The smallest of these, IRP 10, was noteworthy for its strong induction under low iron conditions; whereas it was present in abundance at the low iron concentration, it was virtually absent at the high iron concentration.

B3-306 THE ROLE OF COMPLEMENT RECEPTOR TYPE 3 (CR3; Mac-1) IN ADHERENCE AND UPTAKE OF *MYCOBACTERIUM TUBERCULOSIS*. Colette Cywes, Heinrich Hoppe, Renate R. Scholle and Mario R.W. Ehlers, Department of Medical Biochemistry, University of Cape Town Medical School, Observatory 7925, South Africa

The establishment of an infection by *Mycobacterium tuberculosis* (*M.tb.*) critically depends on the colonization of host mononuclear phagocytes. To this end, *M.tb.* targets specific host cell receptors to promote its adherence and internalization; these receptors include the complement receptors (CR) and the mannose receptor. Of these, CR3 (Mac-1, CD11b/CD18) is of particular interest because it is widely used by diverse pathogens and may confer particular advantages, such as absence of a phagocytic respiratory burst and, potentially, routing into a favourable intracellular niche. At present, the relative importance of CR3 as an *M.tb.* receptor and the dependence on complement (specifically C3bi) for binding are unknown. To address these questions, we have co-transfected CHO cells (as an immunologically "bland", model cell) with the human CD11b and CD18 cDNAs; transfection efficiencies were assessed by immunofluorescence with anti-CD11b and anti-CD18 MAbs. CR3-expressing CHO cells (CHO-CR3) and wild-type cells (CHO-WT) were infected with 7-10 day old cultures of *M.tb.* H37Rv at a ratio of 500:1 (bacteria to cells) in the presence or absence of serum. Quantitation of cell-associated bacteria was achieved by counting under fluorescence microscopy after staining with acridine orange and quenching with crystal violet. The percentage of CHO-CR3 cells infected was consistently 7-fold greater than the CHO-WT cells (78 ± 6 % vs. 12 ± 3 %). These results were identical in the absence of serum. Transmission electron microscopy confirmed that the CHO cells were infected and revealed that in the CHO-CR3 cells the bacilli reside in large membrane-bound vacuoles. These results indicate that (1) CR3 (Mac-1) is a receptor for *M.tb.*; (2) binding to CR3 is complement-independent; and (3) binding to CR3 promotes uptake of *M.tb.* into a non-phagocytic cell by a presumed phagocytic mechanism.

B3-307 **Heterogeneity in peptide processing for recognition by human CD4 T cell clones.** L. Dorrell & A.D.M. Rees, Dept. of G U Medicine, St. Mary's Hospital Medical School, London W2 1NY, UK.

The 19 KDa antigen of M. tuberculosis is secreted during mycobacterial growth and is therefore available for early recognition by CD4 T cells. Early responses may be particularly important in controlling mycobacterial infection and factors which impair the effectiveness of the CD4 T cell response may be important to pathogenesis. A major function of the antigen-presenting cell is to generate the appropriate peptide ligands which, in the context of MHC, are necessary for T cell activation. We have identified heterogeneous behaviour in the capacity of healthy DR3 individuals to present peptide, but not antigen, to 19 kDa-specific DR3-restricted CD4 T cell clones. Proliferation of these clones occurred in response to peptide only when it was presented by autologous APCs. Proliferative response to the antigen MTSE, however, was observed with both autologous APCs and HLA-matched APCs from 5/9 healthy individuals. Aldehyde fixation of autologous APCs abolished the clonal response to peptide but not to antigen. Identical treatment of other DR3 APCs failed to enhance their ability to present peptide. Our observations suggest that the 19.2 peptide is processed by autologous APCs, generating a unique epitope. Heterogeneity in peptide processing may diversify the cellular immune response to mycobacterial antigens.

B3-308 **RESISTANCE TO REACTIVE NITROGEN INTERMEDIATES BY *MYCOBACTERIUM TUBERCULOSIS*.** Sabine Ehrt, Stuart Gunzburg, John MacMicking[1], Carl Nathan[1] and Lee W. Riley. Division of International Medicine, Department of Medicine, [1]Division of Hematology-Oncology, Department of Medicine, Cornell University Medical College, New York, NY 10021

We isolated a *Mycobacterium tuberculosis* DNA fragment that confers a degree of resistance to mammalian cell nitroxergic defenses by constructing a genomic library of strain CB3.3, a clinical isolate of *M. tuberculosis*. The recombinant plasmid, pNO14 confered resistance to reactive nitrogen intermediates (RNI) after transfer to an *E. coli* host. The intracellular survival capacity of *E. coli* HB101 (pNO14) and HB101 (pBluescript) in murine macrophage cell line RAW 246.7 was evaluated. γ-Interferon stimulated macrophages caused inhibition of growth in both *E. coli* strains, but their inhibitory effect on HB101 (pNO14) was 50% less. The specificity of the effect of RNI was assessed by treating the macrophages with *N*-methyl-L-arginine (L-NMA), a substrate analog inhibitor of iNOS (inducible nitric oxide sythase, independent of elevated Ca^{2+}). L-NMA reversed the effect of γ-interferon.

Sequence analysis of the 680 bp *M. tuberculosis* chromosomal DNA fragment in pNO14 revealed an open reading frame of 456 bases (ORF1). Hydropathy analysis of the deduced amino acid sequence of ORF1 showed alternating hydrophobic and hydrophilic regions, highly suggestive of a transmembrane protein.

B3-309 **Early responses of monocytes and T lymphocytes to *M. tuberculosis* and lipoarabinomannan.** Matthew J. Fenton[1], Robin L. Blumenthal[2], Jeffrey S. Berman[1], Hardy Kornfeld[1], and Mary W. Vermeulen[3]. The Pulmonary Center[1] and Dept. of Microbiology[2], Boston University School of Medicine, Boston MA 02118, and Pulmonary Unit, Massachusetts General Hospital, Charlestown MA 02129. A crucial early event in tuberculosis is the ingestion of *Mycobacterium tuberculosis* by alveolar macrophages. This interaction rapidly induces the production of various cytokines that are likely to mediate the destruction of mycobacteria by macrophages. Some of these cytokines are chemotactic polypeptides which function to recruit T lymphocytes to the site of infection. Cytokine production can also be elicited by the mycobacterial cell wall-associated glycophospholipid lipoarabinomannan (LAM). LAM has recently been shown to be released by macrophages containing phagocytosed, but viable, bacilli. Although LAM isolated from avirulent strains of *M. tuberculosis* (e.g. H37Ra) can induce macrophage-derived cytokine production, LAM isolated from virulent strains of *M. tuberculosis* (e.g. H37Rv, Erdman) is chemically distinct and fails to induce cytokine production. Both chemical forms of LAM have been previously shown to be incapable of inducing T cell-derived cytokine (e.g. IL-2, IL-4, LT, γ-IFN) production. We have explored the possibility that virulent and avirulent strains of *M. tuberculosis* elicit different responses from monocytic cells. We futher tested the possibility that LAM can exert biological effects on T cells. Our studies show that viable H37Rv bacilli are more potent inducers of macrophage-derived cytokines (e.g. TNF, IL-1, MCP-1) than either viable H37Ra bacilli or pasteurized (65 °C, 30 min) organisms. Furthermore, purified LAM isolated from both virulent and avirulent strains of *Mycobacteria* was found to induce T cell migration in a modified Boyden chamber migration assay. This latter observation is particularly novel since LAM has not been previously shown to induce biological responses in T cells.

B3-310 TNF-α IS ESSENTIAL FOR PROTECTION AGAINST *M. TUBERCULOSIS* INFECTION IN MICE, JoAnne L. Flynn*, Marsha M. Goldstein†, Karla J. Triebold‖, and Barry R. Bloom‖. Using two experimental murine models for tuberculosis, neutralization of TNF-α in vivo by monoclonal antibody and mice in which the gene for the 55KD TNF-receptor was disrupted, we have demonstrated that TNF-α is required for protection against *M. tuberculosis* infection. Mean survival time for anti-TNF MAb-treated mice and TNFRp55-/- mice was 22 ± 2 days post-infection, while control mice survived >140 days. In addition, bacterial numbers in the organs of the TNF-inhibited mice were 10-100 fold higher than in control mice. Granuloma formation was slightly delayed in the TNFRp55-/- mice, but by 14 days post-infection numbers of granulomas were similar to control mice. However, granuloma composition differed markedly between TNFRp55-/- and control mice. Most strikingly, the granulomas in TNFRp55-/- mice appeared to lack epithelioid cells and were obviously deficient in the ability to control *M. tuberculosis* replication. The production of reactive nitrogen intermediates (RNI) by macrophages, essential for survival in M. tuberculosis-infected mice, was delayed in the TNF-neutralized and TNFRp55-/- mice, compared to wild type mice. This suggested that one possible mechanism by which TNF-α mediates protection against *M. tuberculosis* infection in mice is by the early production of RNI by infected macrophage. These data also demonstrated that TNF-α-independent mechanisms of macrophage exist in vivo. Finally, extensive tissue necrosis, often attributed to TNF-α in tuberculosis, was observed in both TNF-neutralized and 55 KD TNF receptor-deficient mice. These results suggested that TNF-α is not solely responsible for necrosis and tissue damage in tuberculosis, and that other host or bacterial factors, independent of TNF-α, may also contribute to the pathology of this disease.

B3-311 DIFFERENTIAL EXPRESSION OF MACROPHAGE GENES FOLLOWING PHAGOCYTOSIS OF MYCOBACTERIA, Eduardo A. Garcia-Zepeda*#; Jean L. Patterson*.# and Robert H. Husson*. Departments of Infectious Diseases, Children's Hospital * and Microbiology and Molecular Genetics#, Harvard Medical School, Boston MA 02152

The mechanisms used by mycobacteria to survive and replicate intracellularly within the macrophage are not well understood. We have used differential display PCR as a tool to identify genes that are differentially expressed during the phagocytosis of mycobacteria. Two mycobacterial strains, *M. bovis* (BCG strain) and *M. avium* (clinical isolate) were used to infect a murine macrophage cell line (J774). mRNA from infected and uninfected macrophages were isolated at different time intervals after phagocytosis and subjected to differential display PCR. Oligo $dT_{12}NN$ and random 10mer primers were used for PCR amplification of cDNA. Macrophage gene candidates were subjected to further re-amplification by PCR and analyzed by electrophoresis. The expression of a cDNA fragment of a molecular size of 250 bp, clone 62.2, was semiquantitatively analyzed by slot blotting and Northern blotting . Upregulation of this gene was observed within the first 60 min. after infection. The levels of expression were maintained equally after 72h. Further characterization of 62.2 cDNA by sequencing showed 98% homology at the level of nucleotide and amino acid sequence with the A1 gene, a murine macrophage gene previously reported to be involved in early cell activation. A1 has also been shown to be homologous to the *bcl*-2 gene family. Differential expression at the protein level was investigated by western blotting analysis. The ability of LAM, a proposed virulence-associated cell wall bacterial component on modulate the expression of A1 gene is being investigated. A1 may play an important role in triggering macrophage activation mechanisms following phagocytosis of mycobacteria.

B3-312 MYCOBACTERIA AND THEIR ESCAPE FROM IMMUNITY, Barbara C. Hall, and Thomas M. Shinnick, Department of Microbiology and Immunology, Emory University School of Medicine, and Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30329

Mycobacterium tuberculosis and *Mycobacterium leprae* are intracellular pathogens which replicate in macrophages, and it is the pathogen-macrophage interaction which determines the establishment of an infection as well as the host's immune response to the invading mycobacterium. The mechanism(s) by which mycobacteria avoid the bactericidal activities of macrophages is unclear, although many mechanisms have been proposed, including inhibition of phagosome-lysosome fusion and escape from the phagosome into the cytoplasm. To identify the mycobacterial genes and gene products required for intracellular survival, recombinant DNA libraries of mycobacterial DNA in *Escherichia coli* were passed through macrophages to enrich for clones with an enhanced ability to survive intracellularly. Following 3 cycles of enrichment, 13 independent clones were isolated. Three recombinant plasmids were characterized in detail, and each confers significantly enhanced survival on *E. coli* or *Mycobacterium smegmatis* cells carrying them. The nucleotide sequence on two inserts was determined, and one contains and open reading frame which could encode a novel ~15,000 dalton protein.

B3-313 CORRELATION OF CELLULAR AND IGG SUBCLASS RESPONSES IN PATIENTS WITH ACTIVE TUBERCULOSIS. Rabia Hussain Ghaffar Dawood, Mohammed Obaid, Zahra Toossi, Robert Wallis, A. Minai, M. Dojki, Jerrold Ellner. Department of Microbiology, Aga Khan University, Karachi, Pakistan and Division of Infectious Diseases, Case Western Reserve University, Cleveland, Ohio 44106-4984.

Tuberculosis patients with pulmonary (N=138), lymph node disease (N=23) or with both pulmonary and lymphnode involvement (N=6) were assessed for concurrent cellular (DTH skin test and lymphocyte blastogenic responses) and humoral (IgG subclasses) responses. Cellular response to crude mycobacterial antigens (PPD and culture filtrate), and recall antigens (tetanus toxoid and streptolysin O) were significantly suppressed ($p<0.001$) in patients with pulmonary disease. Tuberculous lymphadenitis patients did not show similar suppression of T cell function. It was interesting to note that lymphadenitis patients who also had pulmonary involvement showed a curious dissociation in T cell function. In this group of patients while T cell proliferation and interferon-gamma secretion was similar to the pulmonary group of patients, skin test was similar to the lymphadenitis patients. These results indicate that pulmonary but not the lymphnode disease leads to an overall suppression of T cell memory function. Among the four IgG subclasses, IgG1, IgG2 and IgG3 subclass antibody responses were augmented in all groups of patients (pulmonary and lymph node disease) compared to either healthy endemic controls or household contacts of patients with pulmonary disease with the highest elevation being observed with IgG1 (5.8 times) followed by IgG2 (2.3 times). These results suggests that IgG1 antibody response is independent of both Th1-Th2 responses and may provide a useful marker of clinical disease. (supported by WHO/UNDP Programme for Vaccine Development#V25/181/56).

B3-314 INTEGRATION OF MYCOBACTERIAL LIPOARABINOMANNANS INTO GPI-RICH DOMAINS OF LYMPHOCYTE PLASMA MEMBRANES, Subburaj Ilangumaran, Monique Poincelet, Stephane Arni, Patrick J. Brennan, Nasir-ud-Din and Daniel C. Hoessli. *Department of Pathology, University of Geneva, 1211 Geneva 4, Switzerland and Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA.*

The GPI-linked mycobacterial lipoarabinomannans (LAMs) represent major virulence factors in both leprosy and tuberculosis. They have been shown to induce the production of proinflammatory cytokines in macrophages and downregulate lymphocyte cytokine production. The mechanisms whereby LAMs modulate lympho-monocytic cellular responses at the plasma membrane level have not been studied so far.

We show that both Man- and Ara- LAMs integrate into lympho-monocytic cell plasma membranes and prior deacylation of LAMs abrogates this event, strongly suggesting that integration of LAMs into host cells is mediated by their GPI anchors. Moreover, the basic GPI-hexamannoside (PIM_6) structure common to all LAM molecules specifically inhibits the plasma membrane integration of the high MW LAMs containing elaborate carbohydrate side chains and this inhibitory activity of PIM_6 is abolished by deacylation. The integration of LAMs into isolated plasma membrane fractions enriched in endogenous GPI-linked glycoproteins (Thy-1) is more efficient than in membrane fractions containing both GPI-linked and transmembrane glycoproteins. Our results suggest that LAMs may modulate signal transduction in lympho-monocytic cells by selective perturbation of plasma membrane domains rich in GPI-linked glycoproteins. For instance, incorporation of LAM into murine lymphoma T cells decreases the antibody-induced mobilization of GPI-linked Thy-1 to a larger extent than that of transmembrane CD45. We also suggest that modulation of cellular responses by LAMs will be dependent on the amount of endogenous GPI-linked molecules expressed at the cell-surface of the target cell.

B3-315 IDENTIFICATION AND CHARACTERIZATION OF TWO PHOSPHOLIPASE C GENES FROM *M. tuberculosis* WITH HOMOLOGY TO THOSE FROM *Pseudomonas aeruginosa*. Kristine Johansen, Adriana Vasil, Elise Ross, Ron Gill and Michael Vasil. Department of Microbiology, University of Colorado Health Sciences Center, Denver, CO 80262

The opportunistic pathogen *Pseudomonas aeruginosa* produces a hemolytic (PLC-H) and a nonhemolytic (PLC-N) extracellular phospholipase C that have been implicated in the pathogenesis of pulmonary infections, particularly in patients with cystic fibrosis. PLC-H and PLC-N are highly homologous and both enzymes act on phosphatidylcholine. However, in addition to the differences in their hemolytic activity they have differences in their substrate specificity for other phospholipids. Moreover, while the genes encoding these enzymes respond to similar environmental stimuli there are also differences in their genetic regulation. A recent search of the GenBank database revealed nucleotide sequences reportedly specific to *M. tuberculosis*, and not found in other mycobacterial species, encoding proteins that are highly (approximately 30% identity) homologous to PLC-H and PLC-N. Both of these *M. tuberculosis* genes contain sequences specifying the classical features of a procaryotic signal sequence. One of these *M. tuberculosis* PLC genes, designated *plcI*, has been expressed in *E. coli* using a T7 expression system and the product was demonstrated to have phospholipase C activity. A second partial open reading frame present 3' to *plcI* presumably encodes a second PLC gene, designated *plcII*. Current efforts are aimed at constructing a full-length clone encoding PLCII, determining its complete nucleotide sequence, and whether it too contains PLC activity. The characterization of these PLC genes from *M. tuberculosis* has long term implications for better understanding its pathogenic mechanisms in the lung.

B3-316 CLINICAL AND IMMUNE RESPONSES OF TB PATIENTS TREATED WITH LOW DOSE IL-2, Barbara Johnson and Gilla Kaplan, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY 10021

A potential approach to improving a sub-optimal immune response in TB may be to enhance activation of cytokine genes which promote a maximally beneficial antimicrobial response. We hypothesize that the CMI to TB infection can be modulated by administration of rIL-2, together with multi-drug therapy. Three groups of TB patients could potentially benefit from immunotherapy with rIL-2: those with overwhelming disease not responding to drug treatment and who often manifest antigen-specific immune anergy; patients with multi-drug resistant TB; and patients who are co-infected with HIV-1 associated with reduced cellular immunity.

We have preliminary data from two clinical studies to evaluate the use of low dose rIL-2 in addition to multi-drug therapy to optimize the CMI to TB. In the first study, both treated and newly diagnosed TB patients were given low dose rIL-2 intradermally for 30 days. We next evaluated IL-2 therapy in patients with multi-drug resistant TB to assess the ability of IL-2 to accelerate the slow course of clearance of drug resistant organisms. Patient symptoms including cough, sputum volume, weakness, night sweats, anorexia, and fatigue were observed and given a numerical score daily. To investigate cytokine induction patterns and how these are modulated during therapy, we used quantitative RT-PCR to analyze cytokine mRNA induction in freshly-explanted cells from the peripheral blood, cells isolated from biopsied PPD-reactive sites, and alveolar macrophages from BAL. Plasma samples were used to quantitate levels of circulating cytokines by ELISA.

The effects of IL-2 therapy on bacterial load, patient symptoms, and extent of pulmonary disease will be presented. Levels of mRNA for IL-1β, TNF-α, IL-2, IL-4, IFN-γ, IL-10, and IL-12 in cells from the blood and tissue sites taken before, during, and after IL-2 administration will be correlated to observed symptoms and responses of disease. These observations contribute to our understanding of which cytokines are activated by infection and how levels of cytokine expression are modulated during multi-drug therapy.

B3-317 INTERACTION OF D-COLLAGENS WITH MYCOBACTERIA, K. Joiner[1], V. Polotsky[1], J. Greenberg[1], D. Resnick[2], M. Krieger[2], R.A.B. Ezekowitz[3], and J. Belisle[4], Yale Univ. School of Medicine[1], Massachusetts Institute of Technology[2], Harvard Medical School[3], and Colorado State University[4].

D-collagens are proteins with short collagen domains which participate in innate microbial recognition. Members of the D-collagen family include serum mannose binding protein (MBP), pulmonary surfactant apoproteins A and D, complement component C1q and the macrophage scavenger receptor (MSR). We investigated binding of MBP and MSR to *M. tuberculosis* (Mtb) and *M. avium* (Mac). Both the soluble, recombinant type I isoform of the bovine scavenger receptor (bSRI) and recombinant human MBP bound to Mtb strains H37Rv and Erdman, and to Mac serovars 1,2, 4 and 8. MBP binding was predominantly through the non-collagenous carbohydrate recognition domain (Ca++ dependent, inhibited by mannan), whereas MSR binding was mediated by the collagen domain (inhibited by poly G). To define the mycobacterial ligands for these D-collagens, cell wall lipid fractions and purified components from Mtb and Mac were tested as inhibitors of MSR binding to solid phase scavenger receptor ligands, and as direct ligands for MBP by ELISA. Sulfatides from Mtb were the best inhibitor of MSR binding, consistent with the polyanionic character of MSR ligands. The MSR ligand on Mac is not yet defined. MBP ligands, in descending order of binding, were mannosyl-lipoarabinomannan, lipomannan, phosphatidylinositol mannosides, arabinosyl-lipoarabinomannan, and cord factor (no binding was detected to glycopeptidolipids of Mac), consistent with the known structural requirements for MBP binding (Weis, et. al. Nature, 360:127, 1992). MBP directly opsonized Mac for phagocytosis by PMN, and enhanced C3 deposition on the bacterial surface during incubation in serum. Although both the MSR and MBP have short collagen domains, and both bind to Mtb and Mac, the domains of the proteins which bind to the bacteria and the microbial ligands recognized are different.

B3-318 **ISOLATION OF A PUTATIVE HEMOLYSIN GENE FROM *MYCOBACTERIUM TUBERCULOSIS*,** C. Harold King and Thomas M. Shinnick, Division of Bacterial and Mycotic Diseases, CDC, Atlanta, Georgia, 30333

Hemolysins are known to be important virulence factors of many intracellular bacterial pathogens.We have previously shown that the virulent strain of *Mycobacterium tuberculosis*, H37Rv, produces contact-dependent hemolysis of sheep erythrocytes. Importantly, the hemolytic activity of strain H37Rv is produced also against human erythrocytes, and clinical isolates of *M. tuberculosis* produce this hemolytic phenotype. To isolate the gene that encodes this phenotype and to study the role of the hemolytic activity in the virulence of *M. tuberculosis*, we used the polymerase chain reaction and oligonucleotides corresponding to a highly conserved amino acid sequence of three gram-positive hemolysins to identify a hemolysin homolog from the hemolytic strain of *M. tuberculosis*. Oligonucleotides were designed to amplify a 411-bp region using *M. tuberculosis* codon frequency, and these primers amplified a 400-bp DNA fragment from *M. tuberculosis* genomic DNA. Southern blot analysis of clinical isolates of *M. tuberculosis* and strains H37Rv and H37Ra with the 400-bp PCR fragment demonstrated that all strains contained this DNA sequence on a 2.0-kbp *Sal* I fragment. One open reading frame within the PCR fragment showed 27-30% homology and 41-47% similarity with the amino acid sequence of the three gram-positive hemolysins in the region of conserved amino acids used for oligonucleotide primer construction. The presence of a hemolysin homolog in the genome of *M. tuberculosis* suggest that hemolysin activity may be an important virulence factor.

B3-319 STIMULATION OF MOUSE SPLENOCYTE GAMMA INTERFERON (IFNγ) PRODUCTION BY AN ANTINEOPLASTIC, POLYSACCHARIDE-RICH EXTRACT OF MYCOBACTERIUM BOVIS BCG, Melvin E. Klegerman, Amena S. Muhammad and Michael J. Groves, Institute for Tuberculosis Research, University of Illinois at Chicago, Chicago, IL 60607

An aqueous extract (PS1) of Tice®-substrain BCG, largely comprised of glucose-, arabinose- and mannose-containing polysaccharides, has been shown to antagonize the growth of two murine tumor lines, the S180 sarcoma and the MB49 bladder carcinoma, in vivo and to stimulate incorporation of ^{3}H-thymidine into DNA by both human and murine lymphocytes. Splenocyte cultures were prepared from female CFW mice that were either previously untreated or inoculated 8 days earlier with BCG or PS1. Cultures were treated in vitro with 10μg PS1 or diluent only (controls). PS1 induced secretion of IFNγ to levels of 16ng/mL after 48 hrs, while controls showed only transient, insignificant production of IFNγ. In vivo PS1 pretreatment doubled the IFNγ response, while BCG pretreatment inhibited it by more than 90%, supporting previous evidence that BCG contains both immunostimulatory and immunosuppressive factors. PS1 also caused proliferation of PS1-primed splenocytes in vitro, confirming previous indications that it may also act as a lymphocyte mitogen. Mitogenesis, however, did not correlate with IFNγ stimulation. Isolation of the PS1 immunostimulatory component may help identify mycobacterial cell wall structures that contribute to a protective immune response against tuberculosis.

B3-320 LYSIS OF HUMAN MACROPHAGES BY CYTOLYTIC CD4+ T CELLS FAILS TO AFFECT SURVIVAL OF INTRACELLULAR *MYCOBACTERIUM BOVIS*-BCG.

Dinakantha S.Kumararatne, Nadeem Fazal, David A. Lammas, Mac, Rahelu, Alan D.Pithe[2], & John S.H. Gaston[3]. Departments of Immunology, Infection[2], & Rheumatology[3], University of Birmingham, Birmingham U.K.

Human CD4+, mycobacteria-specific, cytolytic T-cell clones were used to lyse BCG-infected monocyte-derived macrophages, and the effect on the subsequent growth and viability of the organisms was examined. The survival of released bacteria following cell lysis was assessed by both [^{3}H] uridine labelling and colony forming unit (CFU) estimation. The results indicate that even when effective antigen-specific or lectin-mediated cytolysis of the infected macrophages was achieved, there was no evidence for a direct mycobactericidal effect on the intracellular bacteria. This remained the case even if the period of co-culture of T-cells and macrophages was extended up to 48 hours. Pre-treatment of the macrophages within interferon-gamma (IFN-γ) moreover was not able to act together with T cell-mediated lysis to produce inhibition of mycobacterial growth.

B3-321 PHAGOCYTOSIS OF *M. TUBERCULOSIS* BY HUMAN MACROPHAGES IS ACCOMPANIED BY ACTIVATION OF PHOSPHOLIPASE D. David J. Kusner and Larry S. Schlesinger, Dept of Medicine, VA Med. Ctr. and the Univ. of Iowa, Iowa City, IA 52242

Complement receptors mediate the phagocytosis of *Mycobacterium tuberculosis* (*M.tb*) by macrophages. However, little information is available on the macrophage signal transduction pathways which are activated upon phagocytosis of *M.tb*. We have analyzed the activation of phospholipase D (PLD) during phagocytosis of *M.tb* (H37Ra strain) by human monocyte-derived macrophages (MDM). Phagocytosis of *M.tb* in the presence of non-immune serum was accompanied by marked activation of PLD, measured as a 7.6-fold increase over control (SEM=0.84, n=11) in the accumulation of the unique PLD reaction product, phosphatidylethanol (PEt), which occurred in the presence of 0.5% ethanol. Phosphatidic acid (PA), the physiologic product of PLD catalysis, was similarly increased (and the accumulation of PA and PEt were inversely related, dependent on ethanol concentration), confirming a PLD-type enzyme activity. Stimulation of PLD occurred within 5 min of addition of *M.tb*, and accumulation of PEt was maximal at 30 min. A potential role for protein tyrosine kinases (PTKs) in the activation of PLD that accompanies phagocytosis was suggested by inhibition of PEt accumulation by the PTK inhibitors genistein and herbimycin A. Genistein inhibited PLD activity stimulated by *M.tb* by 52% (range 36-64, n=3), and herbimycin produced an inhibition of 79% (range 72-86, n=3). Inhibition of PLD activity by genistein and herbimycin was accompanied by inhibition of phagocytosis of *M.tb* by MDM; 50% inhibition for genistein and 68% for herbimycin. In addition, a competitive inhibitor of PLD, 2,3-diphosphoglycerate, produced a dose-dependent inhibition of phagocytosis, with maximal reduction of 72%. This study demonstrates that PLD is activated during phagocytosis of *M.tb* by human MDM and that this activation of PLD likely involves PTKs. Since down-regulation of PLD activation correlates with an inhibition of *M.tb* phagocytosis, this signal transduction pathway may play an important role in macrophage phagocytosis of *M.tb*.

B3-322 EXPRESSION IN *E.coli* OF A SPECIES-SPECIFIC PROTEIN OF *Mycobacterium tuberculosis* WITH SEQUENCE AND FUNCTIONAL SIMILARITY TO BACTERIAL PHOSPHOLIPASE C. Sylvia C. Leão*, Claudia L. Rocha, Luís A. Murillo, Carlos A. Parra and Manuel E. Patarroyo. *Disciplina de Microbiologia, Escola Paulista de Medicina, São Paulo, Brasil.

One of the major objectives in mycobacterial investigation is the elucidation of mechanisms used by *Mycobacterium tuberculosis* to produce disease. It is possible to speculate that species-specific proteins may be implicated in the unique pathogenic mechanisms characteristic of this bacillus. In previous studies a species-specific 3.0 kb chromosomal fragment was identified from a *M. tuberculosis* library (Infect. Immun. 59:3411-4317,1991). The nucleotide sequence of this 3.0 kb insert was obtained and shown to carry two open reading frames (ORFs) of 560 and 264 amino acids. The putative gene products of these ORFs share a high degree of similarity between each other (68.9% identity) and with two phospholipase C enzymes from *Pseudomonas aeruginosa* (33.3 -39.7% identity). A PCR amplified fragment containing the major ORF was cloned into the pGEX5T expression vector. Cell extracts of *E.coli* over-expressing this glutathione S-transferase fusion protein were shown to produce hemolysis suggestive of phospholipase activity. Phospholipase C has been described as an important virulence factor in *P. aeruginosa* and also in the intracellular pathogen *Listeria monocytogenes*. In this context, it is possible that the proteins identified in this study could also play a role in sustaining tuberculosis infection in humans.

B3-323 CHARACTERIZATION OF MACROPHAGE-INDUCED PROTEINS OF *MYCOBACTERIUM TUBERCULOSIS*, Bai-yu Lee and Marcus A. Horwitz, Department of Medicine, University of California, Los Angeles, CA 90024.

To develop new strategies for combating tuberculosis, we need to learn more about the intracellular biology of *Mycobacterium tuberculosis* in its host cells and about the interaction of *M. tuberculosis* with the host immune system. Toward this end, we have sought to identify *M. tuberculosis* proteins selectively induced upon macrophage infection. Those proteins may be potential virulence determinants or important in the cell-mediated immune response to *M. tuberculosis* infection.

To identify macrophage-induced proteins, we infected THP-1 cells, a human macrophage line, with virulent *M. tuberculosis* Erdman strain. We then labeled bacterial proteins with [^{35}S]methionine while inhibiting host cell protein synthesis with cycloheximide. For purposes of comparison, we also labeled proteins of *M. tuberculosis* growing extracellularly in broth culture in the presence or absence of various stress treatments including heat-shock, low pH, and H_2O_2. The labeled proteins of *M. tuberculosis* growing intracellularly or extracellularly were then separated by two-dimensional gel electrophoresis and quantitatively analyzed by phosphor-Imager. These analyses indicated that the expression of *M. tuberculosis* proteins in broth culture was altered by stress conditions. About half of the proteins present under normal conditions of broth culture were induced or repressed under stress. The pattern of induced and repressed proteins was unique to each stress condition. During infection of macrophages, expression of at least 18 *M. tuberculosis* proteins is induced and expression of 28 proteins is repressed. Of the 18 macrophage-induced proteins, 9 are absent under normal broth growth conditions and 6 are absent under both normal and stress conditions.

B3-324 Genetic analysis of potential attenuating deletions in the BCG vaccine strain. Gregory G. Maharias, Peter Sabo, Mark Hickey and C. Kendall Stover. PathoGenesis Corp. Seattle WA

The attenuated Bacille Calmette Guerin (BCG) strain of Mycobacterium bovis (MBV) has been used as a live vaccine against M. tuberculosis (MTB) for decades. The original BCG strain which was developed by multiple (230 times) serial passages in culture has not been shown to revert to virulence in animals. The attenuating mutations in BCG have not been identified but are suspected to be deletions and/or multiple mutations due to BCG's inability to revert to virulence. We performed genomic subtractions between BCG and MBV/MTB to identify genomic regions of difference between avirulent BCG and virulent MBV(or MTB). The subtracted probes identified four different regions from a cosmid library (22 of 600 clones) of MBV genomic DNA. Sequence comparisons between BCG and M. bovis showed that the three large regions are 12 kb, 11 kb and 8 kb respectively. The analysis of a smaller fourth region is ongoing. Sequence analysis of region 1 (12kb) failed to reveal any significant homologies to known sequences; however the deletion resulted in the premature termination of a large open reading frame. Analysis of region 2 showed that this deletion contained the previously described MPT64 gene, a novel repetitive element, a LysR family regulatory gene and several other notable homologies. Interestingly, the sequence immediately adjacent to region 2 in BCG form a highly stable stem loop structure possibly the result of a transposition/excision event. The third region in MBV also contains a novel repeat motif. To test whether any of these deletions may be responsible for the attenuated properties of BCG we have constructed recombinant BCG containing these regions. These these recombinant BCG will be assesed for the ability to complement virulence in laboratory animals. The results of these experiments will be presented.

B3-325 DIFFERENTIALLY EXPRESSED GENES OF *MYCOBACTERIUM TUBERCULOSIS*, Barbara J. Marston, Thomas M. Shinnick, Division of Infectious Diseases, Emory University School of Medicine and Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, CDC, Atlanta, GA 30333

The virulence of *M. tuberculosis* (MTb) is due in part to the ability of the organism to survive and replicate within host phagocytic cells. To find genes essential for intracellular survival, we are identifying genes that are specifically expressed during intracellular growth. Our strategy uses a "promoter trap" vector (pCL5) containing a promoterless copy of the gene encoding firefly luciferase and a modified integrase gene and attachment site from mycobacteriophage L1. Sau3a fragments of MTb strain H37Rv chromosomal DNA were ligated into a unique BamHI site immediately upstream of the luciferase gene in pCL5. These plasmids, pCL5, and a positive control plasmid, pCL7, (in which luciferase is expressed from the MTb *cpn*-60 promoter) were electroporated into *E. coli* XL1 and MTb H37Rv. Restriction enzyme analysis of 26 independent *E. coli* transformants revealed that 24 (92%) contained plasmids carrying DNA inserts. Southern blot analysis demonstrated that integrated plasmid DNA contained inserts in 9 (82%) of 11 MTb transformants. Promoter expression in the MTb transformants was assayed by measuring luciferase activity (light production) during growth in liquid medium. Strains carrying no plasmids and those containing pCL5 produced <0.002 relative light units [RLUs] per 10^6 cells, whereas strains containing pCL7 produced >0.2 RLUs per 10^6 cells. Among 49 MTb transformants assayed, 42 produced <0.1 RLUs, 5 produced 0.1 to 0.2 RLUs, and 2 produced >0.2 RLUs. We will next identify transformants producing light during growth in macrophage cultures, but not in liquid media, and characterize the DNA inserts (and associated genes and gene products) from such transformants. Genes that are expressed only during intracellular growth may encode critical virulence factors of MTb and are candidates for use as drug targets or in subunit vaccines.

B3-326 COMPARING *IN VITRO* MODELS FOR THE STUDY OF *MYCOBACTERIUM TUBERCULOSIS* VIRULENCE, [1]Parmod K. Mehta, [1]C. Harold King, [1]Kristin A. Birkness, [2]James J. Murtagh Jr and [1]Frederick D. Quinn, [1]Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333 and [2]Pulmonary and Critical Care Medicine, Decatur VA Medical Center, Decatur, GA 30033

Mycobacterium tuberculosis establishes infection after inhalation of the bacilli into the alveoli of the lungs. During this process, *M. tuberculosis* resides principally intracellularly, surviving and growing within the host's phagocytic cells, primarily the macrophages. Translocation across the lung epithelium, endothelium and ultimately into adjacent lung tissues occurs by an unknown mechanism. We have developed a human pneumocyte virulence model to study invasion and multiplication of *M.tuberculosis* using type I and II pneumocytes. Our results show that pneumocyte cells were infected with *M.tuberculosis* within 6 hrs. The organisms replicated intracellularly in these cells more than 55 fold within 7 days. In contrast, when J774 macrophages were infected under the same conditions for 7 days, intracellular growth was observed but the increase was only 0.6 logs and that was maximum after 3 days. All of these observations were substantiated by electron microscopy and light microscopy of Ziehl Neelsen stained formalin fixed infected cells. Currently, our lab is engaged in the development of a tissue culture bilayer model containing type I and II pneumocytes, human macrophages and human microvascular endothelial cells to study passage and intracellular growth of the bacterium within a more complex and relevant *in vitro* system.

B3-327 **INVOLVEMENT OF NK CELLS IN NO PRODUCTION INDUCED BY VIABLE *Mycobacterium bovis* BCG IN MICE.** Masao Mitsuyama, Ikuo Kawamura and Jianfei Yang, Department of Bacteriology, Niigata University School of Medicine, Niigata 951, Japan.

NO (nitric oxide) is a mediator which is important in the expression of macrophage antibacterial killing effect and is dependent on the expression of inducible NO synthase (iNOS).

When mouse spleen cells were stimulated with viable *M.bovis* BCG for 48 hrs in vitro, iNOS mRNA was expressed along with the enhanced expression of mRNA for IFN-γ, TNFα and IL-12 as determined by RT-PCR method. In the culture supernatant, nitrite and IFN-γ could be detected. In contrast, stimulation of spleen cells with killed cells of BCG resulted in the expression of TNFα but iNOS and IFN-γ were scarcely expressed. Messenger RNA for iNOS was not observed in peritoneal exudate macrophages after stimulation with viable BCG, suggesting that iNOS expression by spleen cells is not the result of direct stimulation of macrophages by BCG cells. The level of IFN-γ, one of the most potent inducer for iNOS, was approximately 25 U/ml in the culture of spleen cells with viable BCG stimulation, while the level was almost zero in the culture with killed BCG. Stimulation with recombinant IFN-γ at the dose around 25 U/ml actually resulted in iNOS expression and NO production by spleen cells, however, rIFN-γ-induced NO production was significantly enhanced when BCG was added to the culture. This enhancing effect was observed not only by viable BCG but also by killed BCG. Neutralization of TNFα in culture by specific antibody impaired the iNOS expression and NO production. Elimination of NK cells by NK1.1 antibody showed the similar result. These results strongly suggested that NK cells play a critical role in the induction of iNOS probably via production of TNF and that viable BCG is different from killed BCG in the elicitation of NO response of the infected host.

B3-328 THE EFFECT OF THALIDOMIDE ON THE CELLULAR IMMUNE RESPONSE TO MYCOBACTERIA INFECTION IN MICE. André Moreira, Jie Wang, Liana Tsenova, Robert North and Gilla Kaplan. The Rockefeller University,1230 York avenue. NY.NY,10021.* Trudeau Institute, Saranac Lake, NY.

The protective cellular immune response in mice infected with mycobacteria has been shown to be dependent on an intact granulomatous response, which requires local production of tumor necrosis factor α (TNF-α).

We have evaluated the effect of thalidomide, a selective inhibitor of TNF-α on the granulomatous response to mycobacterial infection in mice. Mice were infected intravenously with M.bovis BCG Pasteur strain or by aerosol route with M. tuberculosis Erdman strain or H37Rv. One group of mice received daily sub-cutaneous injections of 30 µg/kg of thalidomide after infection, control group received diluent only. TNF-α levels in serum were measured by ELISA. Mycobacteria in the lungs, spleens and livers were quantitate by platting tissue homogenate on M7H10 agar plates. Tissue samples for histology were collected. Serum TNF-α, observed only in mice infected with the Erdman strain, were reduced in thalidomide treated mice as was TNF-α mRNA expressed in organs infected with all three mycobacterial strains. Thalidomide treatment was associated with a reduction in the number and size of the granulomas. Thalidomide therapy did not increase mycobacteria numbers recovered from infected organs. On the contrary, in BCG infected mice a drug related reduction in CFU in the lungs at day 21 was observed.

These results suggest that thalidomide reduces TNF-α production in vivo without interfering with granuloma formation or exacerbation of infection.

B3-329 ALTERATION OF MACROPHAGE GENE EXPRESSION BY INFECTION WITH *MYCOBACTERIA*, Gerard J. Nau, Patrick Guilfoile, and Richard A. Young, Whitehead Institute, Cambridge, MA 02142 and Susan Kim and Hardy Kornfeld, Boston University School of Medicine, Boston, MA 02188. A hallmark of infection by *Mycobacterium tuberculosis* is the ability to survive and proliferate within macrophages. It is possible that alteration of macrophage gene expression incited by mycobacteria plays a role in permitting intracellular survival; identification of these genes might provide insight to macrophage factors involved in *M. tuberculosis* pathogenesis. The macrophage cell line, J774, was used to clone and identify macrophage cDNAs representative of genes induced by phagocytosis of *M. bovis* BCG and *Escherichia coli*. These cDNAs were used to determine which genes are preferentially induced by exposure to *M. bovis* BCG. Expression of these genes was also evaluated human alveolar macrophage after phagocytosis of *M. tuberculosis*. Among macrophage genes induced by exposure to these bacteria, osteopontin was specifically induced by phagocytosis of *M. bovis* BCG. MIP1α and ferritin were also identified as genes upregulated after BCG phagocytosis. Competitive PCR experiments revealed that osteopontin gene expression was also induced in human alveolar macrophages following phagocytosis of *M. bovis* BCG and *M. tuberculosis*. Osteopontin, a secreted calcium-binding phosphoprotein and a macrophage attractant, may contribute to recruitment of macrophages and to tubercle formation during *M. tuberculosis* infection. This process of differential screening continues in an effort to identify other macrophage mRNA with altered expression after infection with BCG. Ultimately, alteration of macrophage gene expression may provide a method for identifying specific mycobacterial virulence genes.

B3-330 IDENTIFICATION OF A *SECA* GENE FROM *MYCOBACTERIUM TUBERCULOSIS*. Marie U. Owens[1], C. Harold King[1], Michael G. Schmidt[2], and Frederick D. Quinn[1]. Emerging Bacterial and Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333[1], and Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425[2].

Universally useful vaccines and diagnostic tests for active tuberculosis are currently unavailable. The development of more appropriate candidates is critical, especially with the current rapid spread of the disease world-wide. Good vaccine and probe candidates may be proteins exported to the extracellular environment where these compounds would be exposed to the immune system.

Bacterial protein export is generally mediated by a seriees of secretory (*Sec*) proteins. The *Sec* system may be critical to the virulence of an organism because proteins which serve as sensors of the extracellular environment, secreted toxins or many other proteins that are exported to the medium or bound on the external membrane, are produced within the cell cytoplasm and require public export machinery to determine their proper extracytoplasmic location. Bacterial protein secretion has been defined in *Escherichia coli*, yet recently homologs for several *Sec* factors, including *SecA*, have been identified in Gram positive species as well. Recently, we have identified a *secA* homolog from *M. tuberculosis*. A gene corresponding to the *secA* gene from *E. coli*, particularly homologous in the known prokaryotic conserved regions, has been cloned and sequenced. We are examining the *secA* gene as well as the identity of proteins secreted through this system.

B3-331 *MYCOBACTERIUM HAEMOPHILUM*: A MODEL SYSTEM FOR THE STUDY OF *M. TUBERCULOSIS* CYTOTOXICITY AND INTRACELLULAR PATHOGENESIS. Frederick D. Quinn[1], Laura J. Fischer[1], Elizabeth H. White[2], and C. Harold King[1]. Emerging Bacterial and Mycotic Diseases Branch, Division of Bacterial and Mycotic Diseases[1] and Scientific Resources Program[2], National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333

Mycobacterium haemophilum is a fastidious slow-growing mycobacterium which is emerging in the United States as a serious pathogen among the immunocompromised populations. Of additional importance, this organism replicates intracellularly and produces a cytotoxic phenotype which makes it a potential surrogate for the study of *M. tuberculosis* virulence. During the infectious process, *M. tuberculosis* resides principally intracellularly, surviving, growing, disseminating by residing within the host's cells, and ultimately destroying those same host cells. Thus, a key step in elucidating the disease-causing mechanisms will be the identification and characterization of genes and gene products required for the survival and growth of the bacterium within host cells. As was previously observed with *M. tuberculosis*, we have determined that *M. haemophilum* attaches, invades and multiplies intracellularly within a variety of cell lines, particularly of epithelial origin. Using a multiplicity of infection of 10:1 (bacteria:host cells) we have observed a > 100 fold increase in intracellular bacteria after six days of incubation. Through electron microscopic observations the bacteria appear to remain in vacuoles and not disseminate into the cytoplasm. By 96 hours after infection, the host cells began to lyse, with a majority of the culture destroyed by eight days after infection. Like *M. tuberculosis*, *M. haemophilum* demonstrates a contact hemolytic phenotype when incubated in the presence of washed rabbit erythrocytes. However, unlike *M. tuberculosis* which has a temperature optimum of 37° C, most intracellular replication and cytotoxic destruction by *M. haemophilum* occurred at 33° C. In addition to the hemolysin and other potential toxins, we are currently identifying the genes and gene products expressed by *M. haemophium* under the most destructive conditions in the model system and comparing these results to the factors identified from *M. tuberculosis* infections.

B3-332 *MYCOBACTERIUM MARINUM* AS A MODEL SYSTEM FOR MYCOBACTERIAL PATHOGENESIS, Lalita Ramakrishnan and Stanley Falkow, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305-5402

To overcome the difficulties associated with *M. tuberculosis*, we have selected the human and animal pathogen, *M. marinum* as an experimental model system. *M. marinum* grows optimally at 33°C and hence gives a systemic tuberculosis-like disease to cold blooded animals and a peripheral disease to warm blooded animals, including humans. *M. marinum* has a rapid generation time (4 hours) and can be safely handled using basic bacteriolgical precautions. We have demonstrated that it replicates in eukaryotic cells, in vitro, in a temperature-restricted fashion and that its replication is associated with an easily discerned cytopathic effect. In addition, we have developed a laboratory animal model for chronic disease in *Rana pipiens*. We have also developed genetic tools in *M. marinum*, including genetic transformation and random transposon mutagenesis We will screen the transposon bank generated for mutants defective in persistence and cytopathicity. We have also made a cosmid library of *M. marinum* in the nonpathogen, *M. smegmatis* and are screening it for clones persistent both in the in vitro assay as well as in animals. From this library, we have isolated the gene encoding photochromogenicity in *M. marinum*, and are in the process of characterizing it. We are using this gene as a phenotypic marker to examine the frequency of homologous recombination in *M. marinum*. The combination of these approaches should yield an understanding of factors responsible for persistence and disease.

B3-333 OXIDATIVE STRESS AND THE OXYR RESPONSE IN MYCOBACTERIA, David R. Sherman, Peter Sabo, Taraq Arain, Anna Resconi and C. Kendall Stover, PathoGenesis Corporation, Seattle, WA 98119

All aerobic organisms must protect themselves against toxic oxygen metabolites. This requirement is especially urgent for pathogens such as *Mycobacterium tuberculosis* (MTB) that reside within peroxide-laden macrophages. In gram-negative bacteria, the protein OxyR is a critical component of the response to peroxide. OxyR acts as both a sensor of oxidative stress and a transcriptional activator, inducing expression of detoxifying enzymes such as catalase/hydroperoxidase I (HPI, the *kat*G gene product), alkyl hydroperoxidase (the *ahp*CF gene product) and glutathione reductase (the *gor*A gene product). *E. coli oxy*R-null mutants are 1000-fold more sensitive to peroxide than their congenic parents. To initiate study of this pathway in mycobacteria, we have isolated the *oxy*R homologues of MTB, *M. avium* (MAV) and *M. smegmatis* (MSMG). Surprisingly, the MTB *oxy*R sequence is characterized by several frameshifts and deletions. We have subsequently analyzed *oxy*R sequences from clinical isolates of MTB, compared *oxy*R, *kat*G and *ahp*C transcripts in different mycobacteria and considered the phenotypic response of these germs to peroxide challenge. All three approaches confirm the surprising lack of an oxyR response in MTB or BCG. In contrast, the oxyRs of MAV and MSMG appear functional, suggesting that the loss of oxyR may be unique to MTB/BCG. It is intriguing that this loss correlates with the unique sensitivity of MTB/BCG to the front-line MTB drug isoniazid (INH).

B3-334 REGULATION OF M.BOVIS BCG INDUCED CYTOKINE PRODUCTION FROM MACROPHAGES AND NATURAL KILLER CELLS BY GLUCOCORTICOIDS. Lynette Sigola and Gregory Bancroft. Department of Clinical Sciences, London School Of Hygiene and Tropical Medicine, Keppel Street, London WCIE 7HT.

Several studies have demonstrated that the administration of glucocorticoid hormones causes an increase in mycobacterial organ loads in murine models of infection. Using ELISA assays and RT-PCR qualitative techniques we have investigated the *in vitro* effects of steroids on cytokine production from macrophages and natural killer cells in SCID spleen cell preparations incubated with M.bovis BCG.We have previously shown that BCG causes a dose dependent induction of IFN-g from SCID spleen cell preparations. Using this model we then showed that glucocorticoid hormones cause a dose-dependent reduction in IFN-g production in response to BCG. This effect was specific for the glucocorticoids dexamethasone and hydrocortisone but could not be demonstrated for other steroid hormones such as b-oestradiol, vitamin D3 and dehydroepiandrosterone. Glucocorticoid hormone induced suppression of IFN-g induction was due partly to macrophage inhibition of the production of natural killer cell stimulatory cytokines such as TNF-a. In addition we demonstrated a glucocorticoid inhibitory action on natural killer cells by triggering these cells directly in a macrophage independent manner via exogenous TNF-a and IL-12 in the absence of bacteria; again glucocorticoids suppressed IFN-g induction. With the use of purified peritoneal and bone marrow macrophage populations exposed to BCG we have shown that glucocorticoids also reduce TNF-a production but enhance TGF-b secretion. These results demonstrate that future studies should investigate the effects of glucocorticoids on other macrophage cytokines such as IL-10 and IL-12 to determine if glucocorticoid hormones may suppress T-cell independent murine antimycobacterial immunity by the differential regulation of macrophage and natural killer cell stimulatory and inhibitory cytokines.

B3-335 ANTIMYCOBACTERIAL ACTIVITIES OF *M. LEPRAE* HSP65 REACTIVE T CELL CLONES FROM J774-HSP65 VACCINATED MICE. Celio L. Silva, Marcelo F. Silva, Rosemeire C.L.R. Pietro & Douglas B. Lowrie*, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil, *National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

Mice vaccinated with the single *M. leprae* antigen, hsp65, acquired a high degree of protective immunity against virulent tuberculosis challenge, provided that the antigen was presented to the immune system as an endogenous antigen (C.L. Silva & D.B. Lowrie, Immunology 1994 82 244). Vaccination was by injecting with J774 tumour cells expressing *M. leprae* hsp65 from a transgene (J774-hsp65 cells). Protection could be passively transferred to non-vaccinated mice with either $CD4^+CD8^-$ or $CD8^+CD4^-$ hsp65-specific T cells cloned from the spleens of vaccinated mice (C.L. Silva *et al.*, Immunology, in press). Here we compare the abilities of four $CD4^+CD8^-$ and three $CD8^+CD4^-$ clones with diverse IFN-gamma, IL-4 and cytotoxic capacities to inhibit growth of *M. tuberculosis* in macrophage cultures and to protect non-vaccinated mice. Only supernatants from IFN-gamma-producing clones inhibited *M. tuberculosis* H37Rv uptake of [^{3}H]uracil in bone marrow-derived macrophages and this activity was blocked by mAb against IFN-gamma. Cytotoxic clones that did not produce IFN-gamma could also inhibit [^{3}H]uracil uptake by direct cell/cell contact and this activity was not inhibited by anti-IFN-gamma mAb. The ability of the clones to protect recipient mice (4 weekly iv doses of 5 x 10^6 cells) against challenge infection (cfu in livers 3 weeks after iv challenge with 1 x 10^5 cfu) was most strongly associated with specific cytotoxic capacity and secondarily with IFN-gamma production. The two most protective clones were of $CD8^+CD4^-$ type and strongly cytotoxic. Weekly ip injection of anti-IFN-gamma had minimal blocking effect. In contrast, the mAb treatment blocked the modest protective effect of IFN-gamma +ve $CD8^+CD4^-$ and $CD4^+CD8^-$ clones that had little or no cytotoxic activity. Protection by a IFN-gamma -ve cytotoxic $CD4^+CD8^-$ clone was not blocked with anti-IFN-gamma.

Grants from FAPESP, CNPq and FINEP

B3-336 T-CELL EPITOPES OF THE 30kD ALPHA ANTIGEN OF *MYCOBACTERIUM BOVIS* BCG: POTENTIAL FOR USE IN VACCINES AND DIAGNOSIS, Richard F. Silver, Robert S. Wallis, and Jerrold J. Ellner, Division of Infectious Diseases, The Case Western Reserve University School of Medicine, Cleveland, OH 44124.

Control of tuberculosis is hindered by the lack of both a vaccine of consistent efficacy and of rapid, low technology diagnostic tests for infection with the causative agent, *Mycobacterium tuberculosis*. Culture filtrates of *M. tuberculosis* are protective in both mouse and guinea pig models of tuberculosis. The 30kD alpha antigen, which is the predominant secreted protein in culture filtrates, elicits blastogenic responses in most healthy PPD-positive individuals, but only in a minority of tuberculosis patients. We assessed blastogenic responses of peripheral blood mononuclear cells from twelve healthy PPD-positive individuals to a set of overlapping synthetic 15-mer peptides corresponding to the highly homologous alpha antigen of *M. bovis* BCG (Cambridge Research Biochemicals). Because precursor cells with epitope specificity are infrequent and randomly distributed, we utilized Poisson analysis to assess the blastogenic responses to each peptide at 10μg/ml concentration in twelve replicate culture wells. We identified seven immunodominant regions of the alpha antigen, corresponding to amino acids 1-20, 51-65, 66-85, 131-155, 185-207, 233-238, and 298-311 (from N-terminus). Each subject displayed a positive blastogenic response to at least one of these seven regions. Dominant peptides induced blastogenesis of CD4+ T-cells capable of producing interferon gamma. As a group, these peptides may be useful in the induction of protective immunity against *M. tuberculosis*. Six of the dominant regions are conserved between the alpha antigens of *M. bovis* and *M. tuberculosis*, whereas the remaining region (AA 131-155) differs by a single amino acid substitution. Additionally, two of the dominant peptides differ considerably from the corresponding regions of the alpha antigens of *M. avium* and *M. kansasii*. The differences in sequences may be useful for the development of skin tests of greater specificity than PPD.

B3-337 MECHANISMS OF GRANULOMA FORMATION AND CYTOKINE INDUCTION DURING MYCOBACTERIAL INFECTION OF IMMNUOCOMPETENT AND SCID MICE.
Debbie Smith, Lynette Sigola and Gregory Bancroft. London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, England.

The Severe Combined Immunodeficiency (SCID) mouse mutant compared to its immunocompetent counterpart (BALB\c) provides an ideal model to dissect the immune events necessary for granuloma formation *in vivo*. These mice, which lack T and B lymphocytes exhibit competent innate immune defence mechanisms i.e. macrophages and natural killer cells but are unable to generate organised granulomas during bacterial, protozoal, and helminth infections.

We have followed the outcome of infection with *Mycobacterium bovis* BCG in BALB\c, CBA, C57Bl\6 and SCID mice by histology and CFU counts in the lung, liver, spleen and kidney. In SCID mice tissue counts continued to rise until the twelfth week of infection when the experiment was terminated, while other strains controlled the infection after the third week. Semi quantitative PCR techniques have been used to examine the *in situ* expression of mRNA for a wide range of cytokines including those implicated in macrophage activation, fibrosis and granuloma formation. These included TNF, IFNγ, IL-4, IL-6, GMCSF and IL-10. Qualitative data from immunocompetent mice shows an upregulation in mRNA for IFNγ, IL-12 p40 subunit, GM-CSF and MIP-1α during the first 42 days of infection. Semi-quantitative analysis demonstrates that as bacterial loads begins to fall, infected spleen cells show a clear upregulation of mRNA for IL-10.

We are currently extending these studies to *Mycobacterium tuberculosis* and in addition analyzing localised induction of Class II molecules, recruitment of monocytes and macrophages to granulomatous sites and the altered expression of adhesion molecules during granuloma formation.

B3-338 Protective Immunity: T cell repertoire or T cell function?
Jelle Thole 1,2), Tobias Rinke de Wit 3), Charlotte Hetzel 4), and Jon Lamb 4), 1) Dept . of Medical Microbiology, St. Mary's Hospital Medical School, London, W2 1PG, UK 2) Immunohaematology and Bloodbank, Academic Hospital Leiden, 2300 RC Leiden, The Netherlands 3) Armauer Hansen Research Institute, Addis Ababa, Ethiopia 4) Dept. of Biology, Imperial College of Science, Technology and Medicine, London SW7 2BB, UK.

T cells induced by an infection with mycobacteria are believed to contribute both to elimination of the pathogen and to disease-associated immunopathology. With the objective of defining antigens that are associated with either protection or pathology, we have characterised a panel of T cell antigens of *Mycobacterium leprae* and analysed T cell proliferative responses in healthy infected individuals and patients. The overall similarity of responses in both subject groups -with a frequent recognition of hsp65 kDa and two secreted antigens- suggested to us that the key to distinguishing protective immunity from disease-associated immunopathology may lie in the precise nature of the T-cell response rather than antigenic repertoire. Therefore, we are currently analysing other factors such as the effects of cellular location of antigens, of the viability of the mycobacterial host, and of different capacities of mycobacteria to survive intracellularly on activation of different T cell subsets that are involved in mycobacterial immune responses. To asses the ability of mycobacteria to selectively induce Th1 and Th2 CD4 positive T cell subsets we have expressed model antigens derived from the house dust mite (*Dermatophagoides* spp.) in a variety of mycobacteria. These studies will be important for the evaluation of the application of recombinant mycobacteria as carrier systems, and for the rational design of vaccines against mycobacteria.

B3-339 ACTIVATION OF HUMAN CD8+ T CELLS BY M.BOVIS BCG IN VITRO, Joanne Turner and Hazel M. Dockrell, Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

Studies of the T cell response to mycobacterial infection have predominantly used soluble antigens, such as PPD. This will bias antigen presentation towards presentation by MHC Class II molecules to CD4+ T cells. Recent evidence in the murine system (Flynn *et al*, PNAS 89: 12103, 1992) has shown that mice lacking ß-2 microglobulin and CD8+ T cells cannot control experimental infections with virulent *M.tuberculosis*. We have used live mycobacteria to stimulate human peripheral blood mononuclear cells, in order to assess the extent of activation of CD8+ T cells. Peripheral blood mononuclear cells from BCG vaccinated donors were stimulated *in vitro* with *M.bovis* BCG (Glaxo strain; 5 CFU/macrophage), or with PPD (20μg/ml), for 3, 5 or 7 days. Activation was measured by double staining with antibodies to T cell surface phenotypic markers (CD3, CD4, CD8) and to CD25 as a marker of activated T cells, and the results analysed by flow cytometry. The soluble PPD antigen activated predominantly CD4+ T cells. In contrast, both the number of CD8+ T cells, and the proportion of CD8+ cells expressing CD25 was higher following stimulation with live *M.bovis* BCG. At 7 days, whereas the % of activated CD4+ T cells was two-fold higher in PPD stimulated cultures than in cultures stimulated with *M.bovis* BCG, the % of activated CD8+ T cells was 2.7 times higher in the BCG stimulated cultures. Natural killer (CD16+) cell activation was low after stimulation with both *M.bovis* BCG and PPD. These results indicate that live mycobacteria can activate CD8+ T cells, through presentation of mycobacterial antigens via the MHC Class I pathway, and that this T cell subset may play a role in the immune response to mycobacterial infection in man.

B3-340 MACROPHAGES AND MYCOBACTERIA: CYTOKINES AND EARLY GENE EXPRESSION IN MACROPHAGES IN VITRO

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Macrophages are believed to have a central role in the eradication of bacterial infections, however their role in mycobacterial infections remains controversial. Although it is evident that macrophages can mediate effective anti-mycobacterial responses, there are circumstances where mycobacteria survive and even replicate within macrophages. The factors that determine such phenomena remain obscure.

To further the understanding of the relationship between macrophages and mycobacteria, we have undertaken a study of macrophage responses at the level of gene expression, after exposure to a variety of mycobacterial preparations. Cytokine and early reponse gene expression was determined by RT-PCR. Preliminary results have indicated differential gene responses after exposure to mycobacteria in vitro and in mice after vaccination. In particular live BCG appears to specifically induce higher levels of GM-CSF mRNA when compared to dead BCG, whereas the levels of expression for a range of other cytokines (eg IL-1 alpha and TNF-beta) are similar. Preliminary investigations of the mycobacteria - macrophage interactions during these experiments has also been undertaken at the ultrastructural level.

B3-341 EFFECTS OF AUTHENTIC NITROGEN OXIDES ON MYCOBACTERIA,

Keming Yu, [1]Richard S. Magliozzo, [2]Barry R. Bloom, and John Chan, Departments of Medicine, [1]Molecular Pharmacology, and [2]Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York.

The L-arginine-dependent cytotoxic mechanism of activated murine macrophages is effective in inhibiting and/or killing a variety of microorganisms. We had previously shown that cytokine-activated murine macrophages effectively kill virulent *Mycobacterium tuberculosis*. This mycobacteriocidal activity correlates well with the amount of reactive nitrogen intermediates (RNI) generated. However, the specific RNI species responsible for the observed antimycobacterial effect remains unknown. We have developed an air-tight system in which mycobacteria can be exposed to desired concentrations of authentic NO in an anaerobic atmosphere for various time intervals. In addition, different amount of oxygen can be introduced into the system to determine the effect of NO_2 on mycobacteria. Using metabolic labeling with [^{3}H]-uracil, our results indicate that authentic NO is effectively antimycobacterial in this system. [^{3}H]-uracil incorporation by *M. smegmatis* was inhibited by 100% after five-minute exposure to 760 Torr of NO. 50% inhibition was achieved at approximately 380 Torr of NO. Exposure to anaerobic environment did not affect [^{3}H]-uracil incorporation by *M. smegmatis*. Assessment of the antimycobacterial activity of authentic NO by quantitating colony forming units (CFU) indicate that this nitrogen radical is mycobacteriocidal. Addition of oxygen to the system demonstrated that NO_2 is a more potent antimycobacterial agent compared to NO. [^{3}H]-uracil incorporation by *M. smegmatis* was inhibited by 100% after five-minute exposure to 15 Torr of NO_2. Similar results were obtained for BCG. Our results indicate that: i) both NO and NO_2 are capable of killing mycobacteria, and ii) NO_2 is a more potent antimycobacterial agent compared to NO. The relative significance of these RNI in host defense against *Mycobacterium spp.* remains unkwnon, and is likely to depend on the quantities of these reactive nitrogen species produced at the site of bacterium-host interaction in vivo. The antimycobacteiral effects of these nitrogen radicals on *M. tuberculosis* are currently being investigated.

Epidemiology and Diagnosis of Tb; Application of Molecular Probes; Prospects for The Future: Vaccine Development

B3-400 OPTIMIZATION OF RECOMBINANT MYCOBACTERIAL REPORTER STRAIN FOR DRUG SUSCEPTIBILITY ASSAYS: COMPARISON TO BACTEC AND USE IN INFECTED MACROPHAGES. Taraq M. Arain, Mark J. Hickey, Anna Resconi, Devinder Singh and C. Kendall Stover. PathoGenesis Corporation, Seattle, WA.

Traditional drug susceptibility assays for the slow growing mycobacteria are cumbersome and not amenable to high-throughput drug screening efforts. Luciferase-based reporter gene systems offer substantial advantages but have not been optimized or validated against accepted anti-mycobacterial drug assays. We constructed several integrative and extra-chromosomal firefly luciferase (FF-*lux*) reporter vectors to develop mycobacterial reporter strains optimal for drug susceptibility and high-throughput screening assays. While FFlux expression levels from the *hsp*60 integrative vector chosen (pMV361*lux*) were lower in recombinant BCG (rBCG) than achieved with multi-copy plasmid vectors, assay sensitivity was not a limiting factor and rBCG strains containing pMV361*lux* exhibited more favorable growth characteristics and reproducible luminescence. Seven anti-mycobacterial drugs (isoniazid, ethambutol, streptomycin, amikacin, ciprofloxacin, rifampin and clarithromycin) were tested concurrently against rBCG-pMV361*lux* in the BACTEC and luciferase (Lux) systems over a range of two-fold concentrations. Minimum inhibitory concentration (MIC) determinations were made for each drug in both BACTEC and Lux assays using the 1% resistance criterion. Daily susceptibility data revealed comparable inhibitory activity kinetics and virtually identical MICs for each drug in both assays. The utility of this reporter strain to indicate intracellular anti-mycobacterial activity was also assessed. Using several standard drugs, we demonstrated significant activity versus rBCG-pMV361lux in THP-1 human macrophages within 5 days of infection. These intracellular and microtiter-format *in vitro* reporter strain assays will greatly facilitate efforts to assess the anti-mycobacterial potential of novel lead compounds.

B3-401 PREVENTION OF NOSOCOMIAL TRANSMISSION OF TUBERCULOSIS BY ADMINISTRATIVE CONTROLS. HM Blumberg, D Watkins, P Moore, JE McGowan, Jr., Division of Infectious Diseases, Emory University School of Medicine, and Grady Memorial Hospital, Atlanta, GA, 30303.

A hierarchy of control measures (administrative, engineering, personal respiratory protective equipment) have been recommended to health care facilities by CDC to prevent nosocomial transmission of TB. In addition, OSHA has mandated the use of HEPA-filtered respirators. We evaluated health care worker (HCW) exposure to TB (TB Exposure Episodes and HCW tuberculin skin test [TST] conversion rates) following implementation of expanded TB infection control measures at a public inner-city hospital which each year cares for over 200 patients with laboratory confirmed TB (many of whom are co-infected with HIV). Expanded TB infection control measures, which were instituted after a documented outbreak on two wards of the hospital, consisted chiefly of administrative controls: 1) an expanded respiratory isolation (RI) policy [3/92] which required admitting all HIV-infected patients with an abnormal CXR into a RI room until active TB (or lack of contagiousness) was ruled out by 3 negative AFB smears; in addition, all patients with active TB, TB in the differential diagnosis or AFB sputum cultures ordered were also admitted to RI; 2) increased surveillance; 3) expanded healthcare worker (HCW) education; 4) increasing the frequency of mandatory TST from annual to every 6 month testing. Interim engineering controls (pending completion of renovation of the hospital) consisted of conversion of rooms which have no recirculated air to negative pressure but without 6 air exchanges per hour; in addition a particulate respirator type mask was introduced (6/92) for use by HCWs. The number of TB exposure episodes (i.e., the number of potentially contagious TB patients not placed into RI on admission) decreased from 4.4/mo to 0.6/mo ($p<.001$) after implementation of the expanded infection control measures. In addition, the six month TST conversion rate for HCWs decreased steadily from 3.3% (118/3579; 1/92-6/92), 1.7% (51/2975; 7/92-12/92), 1.4% (67/4715; 1/93-6/93), 0.6% (30/4775; 7/93-12/93) to 0.45% (23/5153; 1/94-6/94) [$p<.001$]. In summary, infection control measures which consisted primarily of administrative controls were effective in reducing nosocomial transmission of TB to HCWs as measured by TST conversion rates at an institution which cares for large numbers of TB patients.

B3-402 T CELL RESPONSE TO *M. TUBERCULOSIS* IN HIV SEROPOSITIVE SUBJECTS; LONGITUDINAL AND CROSS-SECTIONAL ANALYSIS, A.S. De Groot[1,2], K.A. Fisher[1], C.G.P. Roberts[1], G.E. Meister[1], B.T. Edelson[1] K. McGowan[2], J.P. Phair[3], and K. Mayer[1], [1]Division of Biol. and Medicine, Brown University, [2]Dept. Medicine, Lemuel Shattuck Hospital, Boston, MA, and [3] Northwestern University Medical School, Chicago, IL

Objective: To measure T cell responses to a *M. tuberculosis* (Mtb) antigen and to control antigens in peripheral blood mononuclear cells (PBMC) of Mtb and HIV co-infected individuals, and to determine the kinetics of loss of response to Mtb during the course of HIV infection.

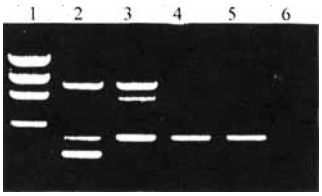
Methods: For the cross-sectional analysis, T cell proliferation and cytokine secretion responses to PPD were measured using PBMC from 25 HIV seropositive and 43 HIV seronegative subjects. Forty of these subjects had been exposed to Mtb, as defined by a positive PPD skin test or history of TB. For the longitudinal study, the *in vitro* responses of PBMC taken from 17 of the study subjects were measured at a minimum of two time points, over a period of 2 to 4 years.

Results: Sixty-seven per cent (12/18) of HIV seropositive, Mtb-exposed individuals did not demonstrate a significant proliferation response to PPD *in vitro* . In contrast, only 5 per cent (1/22) of HIV seronegative, Mtb-exposed individuals did not respond to PPD *in vitro* (POR = 14.7; 95 per cent ci = 4.1 to 52.6). All (7/7) of the HIV seropositive, Mtb-naive individuals failed to demonstrate *in vitro* proliferation responses to PPD, as compared to only 33 per cent (7/21) of HIV seronegative, Mtb-naive individuals (POR = 3.0; 95 per cent ci = 1.5 to 6.2). In the longitudinal study, the T cell proliferation responses to PPD and to the control antigens (TET, PHA, and Candida antigens) were found to vary over time in a non-linear fashion. In some cases, T cell proliferation in response to the test antigens was absent, but was recovered at a later time point. No stable trends in the cytokine secretion responses to the antigens were found.

Conclusions: These results suggest that the increased susceptibility of HIV seropositive individuals to TB may in part be due to impaired T cell responses to Mtb antigens. If, as the longitudinal data suggest, the loss of response to recall antigens such as TET and candida is reversible, this loss of response to Mtb and recall antigens may be due to clonal anergy rather than clonal deletion of antigen-specific T cells. Investigations of means to boost T cell response to Mtb in HIV infected patients may be warranted.

B3-403 MULTIPRIMER PCR SYSTEM FOR THE DIFFERENTIAL DIAGNOSIS OF MYCOBACTERIAL DISEASES. Patricia Del Portillo, Enrique Martínez, Basilio Valladares, Manuel Elkin Patarroyo, Francisco Martín Luengo (R.I.P.), and Manuel Carlos López. Instituto de Inmunología, Hospital San Juan de Dios. Santafé de Bogotá, Colombia and Instituto de Biomedicina y Parasitología "López Neyra", CSIC, Granada, Spain.

A novel multiprimer PCR method has been developed which is able to distinguish, in a one-step reaction, between *Mycobacterium tuberculosis*, atypical mycobacteria and other strains belonging to the *M. tuberculosis* complex. The assay is based on the simultaneous amplification of three different mycobacterial DNA fragments using six different oligonucleotide primers. The first set amplifies a 605 bp fragment from the 32 kDa antigen of *M. tuberculosis*, which is present in most species belonging to the genus *Mycobacteriaceae*. The second set amplifies a 984 bp fragment from the IS6110 repeated sequence of the so-called *M. tuberculosis* complex. The third, derived from the *mtp40* species-specific gene sequence of *M. tuberculosis*, amplifies a 396 bp genomic fragment. This multiprimer reaction results in a unique fragment of amplification from the genomes of atypical mycobacteria, two major amplified fragments from the strains belonging to the tuberculosis complex, and three amplification fragments from *M. tuberculosis*. Results have been obtained using this multiprimer PCR assay with DNA isolated from reference strains, typed clinical isolates, and uncultured clinical samples, demonstrating the potential of this technique in the differential diagnosis of mycobacterial diseases.



1. ΦX174 - HaeIII digest
2. *M. tuberculosis* DNA
3. *M. bovis* DNA
4. *M. avium* DNA
5. *M. gastri* DNA
6. Negative Control

B3-404 MOLECULAR CHARACTERIZATION OF TEXAS ISOLATES OF *MYCOBACTERIUM BOVIS*. Thomas A. Ficht, Allison C. Rice-Ficht, V. Shankar Perumaalla, Stephanie Jones and Michael McMurray. Veterinary Pathobiology, Medical Biochemistry and Medical Microbiology. TAMU and TAES.

Complacency has resulted in minimal research on bovine TB for the last 60 years. The diagnostic skin test used is 100 years old and is unreliable, falsely diagnosing healthy animals or not diagnosing infected animals. Large dairy herds in the El Paso, Texas milkshed cannot eliminate a TB infection that has been smoldering since 1986. Several additional, isolated, severe TB outbreaks in cattle, expanded trade and the immigration of infected farm workers has caused alarm within the state. The rapid expansion of farmed deer and elk industries facilitates dissemination from some infected herds and may facilitate expansion into wild herds. The epidemiological impact of the situation is without parallel. In order to determine the origin(s) and epidemiology of the outbreak we have initiated a study to characterize the *M. bovis* isolates from within the state and in Mexico using molecular techniques. The program is in the preliminary stages and the experimental design is meant to compare the number and location of IS6110 elements, DR- and PGRS- sequences using pulsed-field-gel-electrophoresis and Southern blotting. RAPD-PCR is being used to find sequences which distinguish *M. bovis* and *M. tuberculosis*. To date two arbitrary primers have been identified which reproducibly speciate *M. avium, M. bovis* and *M. tuberculosis*. The primary goal is to determine whether infection originates from multiple sources within or outside the state, or from a single source. To determine the role of wild animals in the spread of disease. Understanding the origin and nature of the disease in Texas is the first step in its elimination.

B3-405 IS*6110* CHROMOSOMAL INSERTIONS AND THE EVOLUTION OF *M.tuberculosis*, Ken J Forbes, Zhigang Fang, Medical Microbiology, Aberdeen University, Foresterhill, Aberdeen, AB9 2ZD, UK.

The population genetics of IS*6110* insertions in specific genomic sites has been determined for 100 strains of *M.tuberculosis* isolated in Scotland which were obtained both from native and overseas patients.

Genomic sequences flanking copies of IS*6110* have been isolated by PCR amplification using primer pairs comprising one IS*6110*-specific oligonucleotide and a second arbitrarily-chosen primer and cycling at lower stringency conditions. By comparison of these complex product patterns with those produced by each primer alone, products which have incorporated one of each primer can be identified. These particular fragments can be specifically re-amplified following extraction of the band from the gel and then sequenced, using the original primers, by cycle-sequencing reactions with fluorescent dye-labelled terminator chemistry. These sequences comprise IS*6110* sequence which runs from the PCR primer to the distal end of the IS and genomic flanking sequence. The first 3nt of this flanking sequence adjacent to the IS derives from the flanking staggered-cut repeat sequence of that IS. A new primer can be designed from these primary sequences which is specific for that sequence and which can be used in conjunction with the IS primer to test the strain collection for the presence of that particular IS copy.

By testing several different strains in the initial PCR screening, selection of IS copies that are not unique to particular strains but are more prevalent in the *M.tuberculosis* population can be made. Flanking sequences from both ends of IS copies can be determined. The linkage of these to previously determined sequences from the other end of the IS is suggested by an identical 3nt direct repeat at the two ends of the IS, and can be confirmed by PCR amplification using the two flanking sequence primers alone.

The distribution in the population of these particular IS copies, their effects on flanking sequences, and their distribution in strains from diverse geographical origins is discussed.

B3-406 A TUBERCULOSIS OUTBREAK IN CUBA WITH A DRUG-SENSITIVE STRAIN ALSO FOUND IN NEW YORK CITY. Friedman CR, Stoeckle MY, Montoro E, Valdivia JA, Diaz R, Kreiswirth B, Riley LW, Murray HW. Cornell University School of Medicine, New York, N.Y. 10021

In contrast with most of the world, Cuba's tuberculosis (TB) rates have declined dramatically over the last 2 decades. Despite deteriorating socioeconomic factors TB case rates have decreased from 11.6/100,000 in 1979 to 5.3/100,000 in 1992. The low HIV-infection rate as well as stringent public health policies may account for this decline in TB.

Recently, a suspected TB outbreak among HIV-infected individuals occurred at The Pedro Kouri Institute (PKI), a tropical and infectious disease referral center in Havana. To determine if a true outbreak occurred IS6110-based RFLP analysis was performed on isolates obtained from patients at PKI from August to December 1993. Isolates from HIV-seronegative patients in Havana during the same time period were also typed.

Twelve isolates were available for RFLP analysis, including 10 from patients at PKI and 2 from out-patients in Havana. Eighty percent of the PKI patients were under 30 years of age (range 21 to 40). The male to female ratio was 3:2. All patients received BCG at birth and at 10 years of age. All TB cultures were isolated from sputum. Only 30% had positive AFB smears. All strains were drug-sensitive. Ten (83%) of 12 typable isolates had identical RFLP patterns. All 10 were from patients hospitalized at PKI. Nine (90%) of the 10 PKI patients were HIV-infected, and one HIV-negative patient was a healthcare worker at PKI. Two HIV-negative patients from the community had unique RFLP patterns. Surprisingly, the outbreak strain was also found in 2 of approximately 1000 RFLP typed TB cases in New York City.

These results indicate an outbreak of drug-sensitive TB among HIV-infected patients at The Pedro Kouri Institute in Cuba. The findings suggest that RFLP typing may be useful in tracing international dissemination of tuberculosis strains.

B3-407 STRAND DISPLACEMENT AMPLIFICATION: A NOVEL TECHNIQUE FOR THE AMPLIFICATION OF DNA AND ITS APPLICATION TO THE DIAGNOSIS OF TUBERCULOSIS, Hellyer T.J., Keating W., Spears P.A., Walker G.T., Cave M.D. & Eisenach K.D., University of Arkansas for Medical Sciences, Little Rock, AR and Becton Dickinson Research Center, Research Triangle Park, NC.

Strand displacement amplification (SDA) is a novel technique for the *in vitro* amplification of DNA. Exponential amplification of target is achieved through the activity of two enzymes, a restriction endonuclease and a strand-displacing DNA polymerase which operate at an optimum temperature of 41°C without the need for thermal cycling. Amplification levels in excess of 10^9 can be achieved within two hours.

We report a comparison of SDA- and PCR-based assays for the detection of *Mycobacterium tuberculosis* in clinical samples. Both systems amplify segments of the IS*6110* repetitive element and incorporate internal control molecules which co-amplify with target DNA. Co-detection of signature and target is performed by hybridization with ^{32}P-labeled probes. Treatment of reaction mixtures with uracil DNA glycosylase eliminates contamination with previously amplified product. The analytical sensitivity of both assays is less than 5fg of purified DNA, the equivalent of one *M. tuberculosis* genome, and results can be obtained within one working day.

PCR and SDA provide rapid alternatives to conventional microbiological approaches to the diagnosis of tuberculosis whilst offering similar benefits in terms of enhanced sensitivity.

B3-408 IMMUNOGENICITY OF A TUBERCULOSIS DNA VACCINE CONTAINING GENES ENCODING THE COMPONENTS OF THE SECRETED ANTIGEN 85 COMPLEX, Kris Huygen, Carine Deneef, Annie Drowart,° Donna L. Montgomery*, Paul Vandenbussche, Margaret A. Liu*, Jeffrey B. Ulmer* and Jean Content, Pasteur Institute of Brabant, 1180 Brussels, ° Hôpital Erasme ULB, Brussels, Belgium and *Merck Research Laboratories, West Point, PA 19486, USA.

The fibronectin-binding antigen 85 complex from *M. tuberculosis* is a family of major secreted 30-32 kDa proteins found in mycobacterial culture filtrate and consists of three components i.e. 85A, 85B and 85C. Its powerful IFN-γ inducing properties in PBMC cultures from most healthy individuals infected with *M. tuberculosis* and in spleen cell cultures from mice infected with live *M. bovis* BCG have suggested that Ag85 might be a protective antigen for tuberculosis. In order to test this hypothesis, the approach of DNA vaccination has been investigated. This technique has proven to be very effective in vaccination against a variety of infectious diseases, including those caused by viruses and parasites.

BALB/c and C57BL/6 mice were injected in the quadriceps with plasmid DNA encoding the genes for 85A, 85B and 85C coupled to an eucaryotic secretion signal and under control of the promoter for the early antigen IE1 from cytomegalovirus. Significant IL-2 and IFN-γ levels could be measured as early as two weeks after a single injection of DNA (100 µg) of 85A plasmid in spleen cell culture supernatants from vaccinated mice, restimulated with native Ag85, purified from BCG culture filtrate. Cytokine levels were comparable to those observed after live BCG infection. Using synthetic peptides for restimulation, the immune responses after DNA injection were found to be directed against the same T cell epitopes as during the natural infection. IL-6 levels were only minimally increased. Sera from vaccinated mice contained antibodies against Ag85, predominantly of IgG2a and IgG2b isotypes. These results suggest that immunization with DNA can lead to selective Th1 T helper cell activation. To define the possible protective effects of immune reactivity against Ag85, DNA vaccinated mice will be challenged with *M.tuberculosis*.

B3-409 NEW APPROACH TO THE DIAGNOSIS OF TUBERCULOSIS.

Geir Johannessen[1], Bjarne Bjorvatn[2] and Dag E. Helland[1].
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[2]Center for international health, University of Bergen, Norway

Our present approach is based upon the assumption that specific mycobacterial components are detectable in the concentrated urine of any patient with extensive mycobacterial disease. Of particular interest are *M. tuberculosis* derived lipoarabinomannan (LAM) and glycolipids carrying the terminal trisaccharide 3-0-methylated-Rhap. Such glycolipids have been detected in serum, are not metabolized in man and hence likely to be found in the urine in an antigenically intact form. To detect these antigens we have produced two monoclonals antibodies reacting against LAM both in ELISA and Western blot. In order to have an inexpensive way of producing antibodies, recombinant antibodies are also produced.

B3-410 EVOLUTION OF THE "W" STRAIN IN NEW YORK CITY. Barry Kreiswirth, Pablo Bifani, Bill Eisner, Vivek Kapur[1], Soraya Moghazeh, Michael Lutfey, Vitalia Henriquez, Andrew Moss, and James Musser[1]. PHRI TB Center, NYC, 10016, [1]Baylor College of Medicine, Houston, TX, 77030-3498.

The largest multi-drug resistant spread of a single clone of *Mycobacterium tuberculosis* has caused four documented outbreaks from 1991-1992 in New York City hospitals and State prisons. This strain, the "W" strain, is resistant to the first line antibiotics and nearly always resistant to ethionamide and kanamycin. In a 1992 survey the strain was isolated from 112 patients from 31 New York City hospitals; 82% were HIV+ and at least 56% died. Since 1991, the genotyping of more than 3,100 *M. tuberculosis* isolates from New York City hospitals, by Southern hybridization with *IS6110*, has identified more than 200 patients infected with the W strain. The *IS6110-PvuII* DNA fingerprint patterns, as compared by a scanning densitometry, has identified DNA patterns that differ from the 17-band pattern that defines the W clone. The 15 related patterns, termed W-variants, differ from the standard W pattern on the basis of 1-3 band changes. Together, the variants define a test set of strains to study their genetic relatedness beyond the *IS6110* probing. To evaluate the strains, Southern hybridization with alternative mycobacteria repetitive DNA probes, pulsed field gel analysis and sequence comparison of drug resistance determinants will be evaluated and correlated with the strain's drug susceptibility profile and patient history. The initial patient data, drug susceptibility profile and *rpo*B gene sequence results indicate that at least 6 of the 15 *IS6110* variants are clearly descendants of the W-clone and that the other 9 strains appear to be unrelated. This finding indicates that one should use caution in interpreting *IS6110* fingerprint patterns and with the exception of an identical match, there is no current approach to evaluate the relatedness of strains that differ by a single *IS6110* copy.

B3-411 ANTI-M.TUBERCULOSIS ANTIBODIES IN HIV-TB. Suman Laal[ab], Karen M. Samanich[a] and Susan Zolla-Pazner[ab], [a]Department of Pathology, New York University Medical Center and Manhattan Veterans Affairs Medical Center, New York, 10016.

HIV-infected individuals are at a significantly higher risk of reactivating latent tuberculosis. We have assessed the presence of circulating antibodies to antigens of M.tuberculosis in retrospective sera from HIV-infected individuals who subsequently developed tuberculosis. Sera from HIV seropositive individuals who were also PPD positive or had a history of being PPD positive were also evaluated. All sera were depleted of antibodies to cross-reactive, conserved prokaryotic epitopes prior to testing in sensitive ELISA assays developed in our laboratory. Antibodies to secreted, cell wall associated and cytoplasmic antigens were assessed (kindly provided by Drs.P.J. Brennan and J.T Belisle). Cellular profiles determined at each time point when the sera were drawn were analyzed. Results show that 50-60% sera from HIV seropositive patients had circulating antibodies to M.tuberculosis antigens several months prior to clinical manifestation of TB. Asymptomatic, PPD positive individuals were negative for anti-M.tuberculosis antibodies and 3/9 patients who were currently PPD negative but had been positive in the past, had antibodies. 3/10 PPD negative HIV seropositive individuals also had circulating anti-M.tuberculosis antibodies. There was no correlation between CD4 T cell numbers, or helper/suppressor ratios and antibody production. Results suggest that presence of antibodies to antigens of M.tuberculosis may be useful as markers for reactivating TB.

B3-412 THE RAPID DETECTION OF MYCOBACTERIUM AVIUM IN STOOL SAMPLES USING POLYMERASE CHAIN REACTION, Zhongming Li, Gil H. Bai, Michael J. Brennan and Sheldon L. Morris, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892

Mycobacterium avium is the most frequent bacterial isolate and the most common cause of systemic bacterial infections in patients with AIDS. To improve and accelerate diagnosis of M. avium infections in clinical specimens, a species-specific polymerase chain reaction (PCR) protocol, which utilizes primers designed from the unique 16S rRNA gene sequence of M. avium , has been developed. In addition, an internal control has been generated to detect false-negative PCR reactions. To rapidly detect M. avium in stool samples that contain substantial Taq polymerase inhibitors, this PCR protocol has been coupled with an immuno-magnetic procedure designed to selectively bind M. avium bacilli from human stool samples using magnetic beads coated with an anti-mycobacterium monoclonal antibody. Ten M. avium bacilli can be detected in spiked stool samples using this modified PCR method. Culture confirmed clinical samples from AIDS patients are currently under analysis. The rapid and sensitive detection of M. avium by PCR should facilitate the clinical management of M. avium infections.

B3-413 PROTECTION AGAINST *TUBERCULOSIS* BY IMMUNIZATION OF MICE BY INTRAMUSCULAR INJECTION OF NAKED DNA EXPRESSING *M. leprae* HSP65. Douglas B. Lowrie, M. Jo Colston and Ricardo E. Tascon, National Institute for Medical Research, Mill Hill, London NW7 1AA.

It has historically proved difficult to effectively vaccinate against tuberculosis with mycobacterial components or even with whole dead mycobacteria; protection was always inferior to that obtained with the live attenuated vaccine BCG. We have found that this may no longer be the case. *M. leprae* hsp65 conferred substantial protection to BALB/c mice, but only when it was generated as an endogenous antigen within antigen presenting cells (C.L. Silva & D.B. Lowrie, Immunology 1994 **82** 244). This was achieved by cloning the mycobacterial gene into a retroviral vector, expressing it in J774 macrophage-like tumour cells and injecting the transfected tumour cells iv or ip into the mice. The resulting protective immunity could be passively transferred with antigen-specific T cells, particularly $CD8^+$ T cells, cloned from spleens (Immunology, C.L. Silva *et al*, in press). We now show that simple direct intramuscular injection of plasmid DNA may achieve the same result. The *M. leprae* gene for hsp65 was cloned downstream of the promotor for the murine "housekeeping" gene for hydroxymethylglutaryl-CoA-reductase. 50 μg of plasmid DNA was injected into each hind leg into the quadriceps muscle of groups of 4 outbred Parkes albino mice 4 times at approximately 3-week intervals. Two weeks after the last injection the mice were challenged ip with 2×10^5 viable *M. tuberculosis* H37Rv. 6 weeks later cfu counts in livers and spleens were 10- to 100-fold lower in hsp65 DNA vaccinated mice than in sham-vaccinated (vector DNA only) mice and not significantly different from live BCG vaccinated mice ($p > 0.05$). Genes for other mycobacterial antigens are being similarly tested.

B3-414 DETECTION OF MYCOBACTERIAL ANTIGENS BY PIEZOELECTRIC IMMUNOSENSORS, Candace McCombs[1], Joseph Michalski[1], Steven Perlaky[2], Steven Schreck[2], Michael Neuman[3], Dept of Medicine[1], University of South Alabama, Mobile, AL 36688;[2]S&P Medical, Inc. Mobile, Al; [3]Case Western Reserve University, Cleveland, OH

Piezoelectric immunosensors were constructed by coupling polyclonal antibodies to the gold electrode surfaces of piezoelectric quartz crystals by either of two methods. In the first method, antibody was thiolated with Traut's reagent and covalently linked to the gold surface via a sulfur bond. In the second method, oxidized antibody was covalently linked to a layer of (γ-aminopropyl) triethoxysilane layered onto the gold electrode surface. Experimental crystals were coated with rabbit IgG antibody to M. tuberculosis, while control crystals were coated with an irrelevant antibody, specific for an antigen not present in culture supernatants. Experimental and control crystals were exposed to culture supernatants of attenuated M. tuberculosis organisms for 60 minutes, washed, dried, and the change in resonant frequency measured as a indication of mass gain on the electrode surface.

As shown below, piezoelectric immunosensors constructed by either method showed significantly greater frequency change for crystals coated with mycobacterial antigen-specific antibodies than for crystals coated with an irrelevant antibody.

Antibody attachment	Antibody specificity	No. of crystals	Change in frequency (mean ± SE)	Mann-Whitney test
Traut's	anti-TB	12	523 hz±84	p=0.006
Traut's	control	11	244 hz±55	
via silane	anti-TB	13	470 hz±81	p=0.008
via silane	control	12	193 hz±36	

These results indicate that piezoelectric immunosensors can detect mycobacterial antigens in culture fluids in an immunologically specific manner, in spite of the presence of potential interferents.

B3-415 ESTABLISHMENT AND EVALUATION OF A MULTIPLEX POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF TUBERCULOSIS, Abu S. Mustafa, Athar Ahmed, Adnan T. Abal and Tulsi D. Chugh, Department of Microbiology, Faculty of Medicine, Kuwait University and Chest Diseases Hospital, Ministry of Public Health, Kuwait

We report the establishment of a multiplex PCR assay for specific identification of M. tuberculosis complex and their differentiation from other mycobacteria by a single tube amplification of 131 bp, 240 bp and 383 bp DNA fragments from the target M. tuberculosis genes encoding 19 kDa, MPB64 and 65 kDa proteins, respectively. A 500 bp λ phage DNA was also amplified in the same reaction tube to serve as a positive control and to check for inhibitors in clinical samples. DNA from cultured specimens was extracted by a simple procedure of heating the samples at 95^0C for 15 minutes. The clinical specimens were heated and the DNA was ethanol precipitated. In the multiplex PCR assay, the 383 bp DNA fragment was amplified from all mycobacteria, amplification of the 240 bp DNA fragment was specific to M. tuberculosis complex and M. fortuitum and the 131 bp DNA fragment was amplified from the organisms of M. tuberculosis complex and M. scrofulaceum. None of these DNA fragments were amplified from non-mycobacterial organisms and all the three bands were positive for M. tuberculosis complex. We have evaluated the multiplex PCR in the diagnosis of pulmonary and extra-pulmonary tuberculosis. As compared to culture, the sensitivity and specificity of PCR was 86% and 100%, respectively. However, the culture may take 10 days to several weeks, whereas the results of the multiplex PCR were reported on the next working day.

B3-416 FALSE-POSITIVE CULTURES FOR *MYCOBACTERIUM TUBERCULOSIS*: MOLECULAR ANALYSIS. Susan M. Ray, A. Kshirsagar, B. Metchock, F. Nolte, Division of Infectious Diseases, Emory University School of Medicine and Grady Memorial Hospital, Atlanta, GA 30303.

False-positive cultures for *Mycobacterium tuberculosis (M.tb)* can be a source of clinical confusion and may be more likely to occur in communities where tuberculosis (TB) incidence is increasing and large numbers of patients are evaluated for possible TB. We investigated three groups of patients (A,B, and C) in which one or more of the patients was suspected of having a false-positive culture for *M.tb*. A false-positive culture was defined as a specimen that was submitted from a patient whose clinical course was not consistent with tuberculosis and that grew *M.tb* with the same restriction-fragment-length-polymorphism (RFLP) pattern as that of a true-positive specimen processed concurrently by the laboratory. Patient A1 presented with a rapidly progressive pneumonitis and had AFB found on a concentrated BAL specimen which later grew *M.tb*. After careful evaluation, she was diagnosed with cyclophosphamide pneumonitis and improved with steroid therapy. Patient A2, with classic pulmonary TB, submitted sputum specimens with 4+ smears on the same day that patient A1's BAL fluid was processed. RFLP analysis of A1 and A2's cultures demonstrated matching patterns. Patients B1 and B2 had positive cultures for multi-drug resistant TB (MDR-TB) reported after discharge. Both had recovered from pulmonary illnesses without treatment for tuberculosis. Patient B3 had pulmonary TB (with primary drug-resistance) and submitted sputum specimens on the same day that specimens from B1 and B2 were processed. RFLP analysis demonstrated that B1, B2, and B3 had the same pattern. Patient C1 presented with a large pleural effusion and died on the 10th hospital day. A pleural fluid culture was reported growing *M.tb* after his death. Patient C2 had pulmonary TB and his sputum culture was reported positive within 2 days of C1 although it was processed a week before C1. RFLP analysis demonstrated different patterns for C1 and C2 which was consistent with the fact that there had been no opportunity for cross-contamination. RFLP analysis can provide evidence which supports diagnostic conclusions based on thoughful clinical evaluation. In a community with high transmission rates matching RFLP patterns may be expected and thus clinical evaluation remains as the most important diagnostic tool.

B3-417 ρ-NITRO-α-ACETYL AMINO-β-HYDROXY-PROPIOPHENONE (NAP) DIFFERENTIATES BETWEEN MYCOBACTERIA TUBERCULOSIS COMPLEX AND OTHER MYCOBACTERIA IN THE LUCIFERASE REPORTER PHAGE ASSAY, Paul Riska, °William R. Jacobs, Jr., °Barry R. Bloom and John Chan, Division of Infectious Disease, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY10467, and °Division of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461.

As the incidence of multidrug resistant tuberculosis continues to increase, the need for rapid identification and drug susceptibility testing has become more acute. The luciferase reporter mycobacteriophage can specifically infect viable mycobacterial cells, which leads within hours to the transcription and translation of the luciferase enzyme. In the presence of cellular ATP and the exogenous luciferin substrate, visible light is detected. Effective antinicrobial agents will interfere with light production at any of the stages of this process.

One potential limitation of the first generation phage (phAE40) is that it has a wide host range among mycobacteria. Since more than half of clinical isolates at our hospitals are non-tuberculous mycobacteria, which collectively require less urgent treatment, the ability to rapidly and reliably identify *M. tuberculosis* is critical to the proper management of patients with mycobacterial infections. We have taken advantage of an antibiotic, ρ-nitro-α-acetyl amino-β-hydroxy-propiophenone (NAP, Becton Dickinson), a chloramphenicol derivative that has been extensively evaluated over the last decade in clinical practice, in the BACTEC system for species identification. It has been found to be highly (>99.5%) sensitive and specific, with the literature citing over 6000 isolates tested. We have now demonstrated that NAP can be utilized in combination with the luciferase phage assay to specifically decrease light output from BCG and *M. tuberculosis* Erdman, without decreasing light output from *M. smegmatis* or *M. avium* lab strains. The dose and treatment time optimums have been defined, and suggest a potential time saving over the current BACTEC method. Testing of the applicability of the mycobacteriophage/NAP system to clinical isolates of *M. tuberculosis* and *M. avium*, as well as a panel of "atypical" mycobacteria is currently underway.

B3-418 HUMAN HUMORAL RESPONSES TO M.TUBERCULOSIS. Karen M. Samanich[a], Suman Laal[ab], and Susan Zolla-Pazner[ab], [a]Department of Pathology, New York University Medical Center and Manhattan Veterans Affairs Medical Center, New York, 10016.

Major differences between epitopes recognized by human and animal immune systems have been observed for many organisms. Limited direct analysis of the human humoral immune responses to M.tuberculosis has been done, primarily by one-dimensional SDS-PAGE of M.tuberculosis supernatants. We have defined the breadth of the human humoral response to M.tuberculosis antigens using several different antigen preparations in sensitive ELISAs. The antigens were kindly provided by Drs. P.J. Brennan and J.T. Belisle and included: 1) Total culture supernatants containing secreted antigens (150-200 proteins and LAM); 2) LAM-free secreted antigens; 3) Purified LAM; 4) Total cell walls containing cell wall core, associated proteins and LAM etc.; 5) SDS-soluble cell wall proteins; 6) Purified cell wall core comprised of the mycolyl-arabinogalactan-peptidoglycan complex; 7) Total sonicated extract containing primarily cytoplasmic antigens. Results show that: 1) antibodies to a large repertoire of antigens, i.e. secreted proteins, cell wall antigens cytoplasmic antigens, LAM are present in 80% of the patients; 2) all classes of antigens are recognized by the antibody positive patients 3) patients who lacked antibodies were negative for all the antigenic fractions of M.tuberculosis. Detailed analysis of antibody reactivity will be presented.

B3-419 RAPID SIMULTANEOUS DETECTION AND DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS. Leo Schouls[1], Judith Kamerbeek[1], Miranda van Agterveld[1], Dick van Soolingen[1], Annelies Bunschoten[1], Arend Kolk[2], Sjoukje Kuyper[2] and Jan van Embden[1]. *Unit Molecular Microbiology[1], National Institute of Public Health and Environmental Protection. 3720 BA, Bilthoven and Laboratory of Tropical Hygiene[2], Royal Tropical Institute, 1105 AZ, Amsterdam, The Netherlands.*

Most methods described for differentiation of *M. tuberculosis* depend on restriction fragment length polymorphism and are visualized by Southern blotting. These typing procedures require time consuming, sophisticated laboratory methods because they depend on the availability of purified DNA from cultured *M. tuberculosis* strains. We have developed a method to simultaneously detect and type *M. tuberculosis* from clinical material without the need to culture the organism or purify DNA. The method takes advantage of the DNA polymorphism at a unique locus in the *M. tuberculosis* chromosome containing multiple 36 base pair Direct Repeats (DRs), which are interspersed by non-repetitive unique spacer sequences, each 35-41 bp in length. Because the DR sequence is well conserved among *M. tuberculosis* strains, each DR copy within the locus is a potential target for in vitro amplification by PCR and because of the high copy number in the DR cluster such a PCR is more sensitive than when using a single copy DNA target. The typing method referred to as spoligo typing (spacer oligo typing), relies on the presence or absence of spacer sequences in the amplified DNA which are detected by hybridization of the labeled amplified DNA to multiple synthetic spacer oligonucleotides, which are covalently bound to a filter. In our experiments we used 37 of the 41 spacer sequences present in the DR region of *M. tuberculosis* H37Rv and 6 spacers from *M.bovis* BCG. Because *M. tuberculosis* isolates differ in the presence of spacers sequences, strains can be differentiated based on their hybridization patterns (spoligotypes). Typing of a large number of *M. tuberculosis* strains showed that epidemiologically non-related strains differed in one or more spacers, whereas the isolates from outbreaks were identical. The sensitivity of the PCR with DR as a target allows to detect and type a single cell of *M. tuberculosis*. When the spoligo typing was applied directly on clinical samples it was found that culture positive samples tested in this study did yield spoligo types identical to those obtained from the cultures of the corresponding samples. In contrast to RFLP typing the method of spoligo typing will facilitate large scale DNA typing of *M. tuberculosis*. Strain differentiation by oligo typing may provide important information to establish risk factors for transmission, to determine infection versus reactivation and to determine the efficacy of BCG vaccination to protect against particular subsets of *M. tuberculosis* strains.

B3-420 ARE IS6110 BASED RFLP CLUSTERS OF MTB CLONAL?

Samir P. Singh, Peter M. Small, Division of Infectious Diseases, Stanford University, CA.

Introduction: A **genotype** is a group of isolates which share a genetic characteristic. IS6110 based RFLP analysis of MTB is a genotyping system which has been found to correlate with epidemiology in most instances. A **clone** is a group of isolates which share so many characteristics that the most likely explanation for their similarity is a common lineage. Clonality, being an intrinsic property of epidemiologically connected cases of tuberculosis, may be a superior basis for molecular epidemiology. In the present study, we used a panel of genotyping techniques (**multilocus genotyping**) to asses clonality.

Methods: As part of a two year population based study of tuberculosis in SF, 3 clusters of TB patients with the same IS6110 genotype were identified and intensive epidemiological investigations were conducted in a blinded fashion. A few patients had no discernible epidemiological link with other patients in the cluster. The three clusters - Cluster A (2 copies of IS6110; 23 patients), Cluster B (5 copies of IS6110; 15 patients), Cluster C (9 copies of IS6110; 30 patients) - were genotyped using restriction endonuclease Alu I and Direct Repeats (DR) and Polymorphic GC Rich Repeats (PGCR) as probes.

Results: Genotyping with DR and PGCR divided the three clusters by IS6110 into subclusters. With one exception, the subclusters defined by DR and PGCR typing were identical. Cluster A, disintegrated into 5 subclusters and 1 unique pattern. Cluster B, broke up into 1 subcluster and 1 unique pattern. Cluster C, divided into 1 subcluster and 1 unique pattern. Epidemiologic investigations of Cluster C identified three epidemiologically unlinkable cases. One of the these three corresponded to the unique pattern with DR and PGCR in Cluster C.

Conclusions: Genotyping with IS6110 provides useful information but may introduce a misclassification bias in epidemiologic studies. Genotyping of the three clusters with DR and PGCR provided identical grouping, suggesting a clonal relation among the subclustered isolates. A panel of probes may be necessary to infer a clonal relationship among MTB isolates. Further research is needed to 1) identify and characterize a variety of multilocus genotyping approaches and 2) assessing the population structure of MTB using population biology methods.

B3-421 DEVELOPMENT OF A RAPID GROWTH ASSAY FOR MYCOBACTERIA USING MICROENCAPSULATION ,

Susan J. Sullivan, Colleen Ryan and Peter A. Lopez* One Cell Systems, Inc., Cambridge MA 02139 and *The Core Flow Cytometry Facility, Dana Farber Cancer Institute, Boston, MA 02115.

The recent increase in the incidence of tuberculosis cases as well as the emergence of multidrug-resistant TB strains has become a serious public health concern. Effective control of transmission in vulnerable population groups is dependent on rapid identification of the infectious agent and its drug susceptibility. Timely treatment with the appropriate therapeutic agent is essential both for management of the individual patient and minimizing transmission. However, the slow growth rate of mycobacteria has undermined the ability to quickly identify the antimicrobial resistance. We are developing a mycobacterial growth assay based on microencapsulation technology to be used in conjunction with flow cytometric analysis. In this system, mycobacteria are encapsulated into agarose gel microdrops (GMDs) approximately 30 μm in diameter. Our initial experiments have investigated several different strains of mycobacteria, including M. bovis, smegmatis and kansasii as model systems. We have demonstrated growth of individual microcolonies within several days after GMD encapsulation. Colony growth can be monitored within the GMDs using fluorescence microscopy and/or flow cytometry to evaluate the intensity of colony staining by propidium iodide, auramine and auramine/rhodamine after culture for various times at 37°C. We are also investigating the ability to identify the encapsulated mycobacteria using commercially available probes (GenProbe, Inc.). Encapsulation of mycobacteria allows for quantitative analysis of fluorescence staining as a measure of colony growth using flow cytometry. This has the potential to facilitate rapid and automated evaluation of inhibition of growth by antimicrobial agents and shorten the 4-6 week time frame for analysis of clinical specimens associated with the currently available technology.

B3-422 DIFFERENTIATION OF MYCOBACTERIUM ULCERANS, M. MARINUM, AND M. HAEMOPHILUM BY DNA-DNA HYBRIDIZATION AND 16S rDNA SEQUENCING.

T. Tønjum[*1], D.B. Welty[2], and P.L.C. Small[2]. [1]Bakteriologisk Institutt, National Hospital, Oslo, Norway, and [2]Rocky Mountain Laboratory, Hamilton, Montana.

Mycobacterium ulcerans, M. marinum, and M. haemophilum are slow-growing mycobacterial species with optimal growth-temperatures of 28-31°C. Emerging as clinically significant pathogens, they are mainly the cause of skin infections, but M. marinum and M. haemophilum can also give rise to invasive infections in immunocompromised patients. The three species are closely related, but their exact taxonomic placements have not been precisely determined. We performed total genomic DNA-DNA hybridizations and 16S rDNA sequencing to clarify their relationships to each other and to M. tuberculosis. M. ulcerans and M. marinum were found to be mutually most closely related, also displaying strong affinity to M. tuberculosis. M. haemophilum was more distinct from M. ulcerans and M. marinum, and appeared to be as closely related to M. ulcerans and M. marinum as to M. tuberculosis. These findings are most important with regard to the development of species-specific DNA probes and PCR assays as diagnostic tests for Mycobacterium ulcerans, M. marinum, and M. haemophilum in clinical settings and epidemiological investigations.

B3-423 MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS: STRAIN SUBTYPES IN A VERY HIGH INCIDENCE COMMUNITY,

Paul van Helden, Rob Warren, Madelene Richardson, Ian Wiid, Cedric Werely, Nulda Beyers, Peter Donald, MRC Centre for Molecular and Cellular Biology, University of Stellenbosch Medical School, Tygerberg, South Africa.

DNA fingerprinting is at present being applied to strains of M tuberculosis isolated from a community with an estimated incidence of 1 000/100 000 p.a.

Using IS6110 as a marker, 50% of the strains typed fall into clusters: this being an indicator for cases of recent transmission. The criteria selected for a cluster were defined as 100% pattern-matching. However, the results also show that closely related strains (eg 85% identity) can be seen in individuals who are in close contact with others within a cluster. These strains have clearly undergone one (or more) mutational event, but must be derived from the parental cluster. If these strains are included, clustering rises to 68% in this community. This extremely high figure is perhaps not surprising, given the high incidence community, but is clearly dependent on the definition of a strain.

Using a slightly less sensitive marker than IS6110, viz. $(GTG)_5$, the high degree of clustering can be confirmed, (in excess of 65% clustering). However, using a new polymorphic probe cloned from the genome of M tuberculosis, we can often distinguish between strains within a IS6110 cluster. This applies particularly to low copy-number IS6110 strains. The different markers, useful in cluster analyses, and showing different rates of change within the genome, should be useful in studying the genome (evolution) of the organism and its passage through its host.

B3-424 IDENTIFICATION OF POLYMORPHIC EXPRESSED SEQUENCE TAGS (ESTs) IN VIRULENT VERSUS AVIRULENT STRAINS OF *Mycobacterium tuberculosis* USING RNA ARBITRARILY PRIMED POLYMERASE CHAIN REACTION (RAP-PCR). John R. Webb, Jeffrey A. Guiderian, Steven Johnson and Steven G. Reed, Infectious Disease Research Institute, Seattle, WA 98109

The H37Ra (avirulent) and H37Rv (virulent) strains of *Mycobacterium tuberculosis* provide an excellent model system to study the basis of virulence in these important human pathogens. We have employed the RAP-PCR technique to identify transcripts which are either expressed differentially or contain sequence polymorphisms between these two derivatized strains. A total of eight polymorphic PCR products have been isolated to date, one of which recognizes a differentially expressed transcript when hybridized against Northern blots. The remaining seven products recognize transcripts with similar expression levels and therefore likely represent sequence polymorphisms within the PCR primer annealling site. Partial sequences of four of the polymorphic PCR products have been determined and none of the four exhibit any significant similarity to sequences currently held in the genome databanks. Polymorphisms identified by the RAP-PCR technique will be used to compile a library of candidate virulence factors which may be used individually or in tandem for complementation analysis of the virulent phenotype of *M. tuberculosis*.

Late Abstracts

CELL-FREE SYSTEMS FOR MYCOLIC ACID BIOSYNTHESIS IN *Mycobacterium smegmatis* AND CHARACTERIZATION OF THE STEPS INHIBITED BY ANTIMYCOBACTERIAL DRUGS. J. Lonsdale[1] and G.S. Besra[2]. [1]Department of Microbial Metabolism and Biochemistry, SmithKline Beecham, Brockham Park, U.K., and [2]Department of Microbiology, Colorado State University, Fort Collins, CO 80523 U.S.A.

Cell-free systems have been employed to examine the biosynthesis of mycolic acids in *Mycobacterium smegmatis*. Radiolabelled precursors including acetate and postulated intermediates such as C24:*cis*D5 fatty acid have been incubated with membrane preparations from *M. smegmatis* and their incorporation into mycolate followed using either thin-layer chromatography or precipitation/ filtration based techniques. The incorporation of label into mycolate is dependent upon time, substrate and protein concentrations. This system allows further definition of the steps inhibited by a number of antimycobacterial drugs. Data concerning the points of intervention of both isoniazid and ethionamide and the use of these assays as screens for the discovery of new inhibitors of mycolic acid synthesis will be discussed.

IDENTIFICATION OF AN ELASTASE PRODUCED BY M. TUBERCULOSIS, Sharon S. Rowland, Department of Medical and Research Technology, University of Maryland School of Medicine, Baltimore, MD 21201.

Elastase activity has been identified in culture filtrates of *M. tuberculosis* (Mtb). Elastase is a protease which degrades elastin, the fibrous protein responsible for the lung's elasticity. The production of elastase by a pathogen can be a virulence factor, allowing the organism to invade cells, spread, and cause tissue necrosis, leading in turn to an exacerbated inflammatory response. Elastase has been identified as a virulence factor for two other respiratory pathogens, *Pseudomonas aeruginosa* and *Aspergillus fumigatus*. Four strains of *Mtb* (H37Rv, H37Ra, clinical isolate 1, and clinical isolate 2) were grown as standing cultures in Sauton's or elastin broth at 37°C for 5 weeks. Culture filtrates were incubated with elastin-Congo red in the presence of 0.05M Tris-HCl, 1 mM $CaCl_2$, pH8.1 at 37°C for 16-18 hours. All four strains were positive for elastase activity. The elastase was retained by a m.wt. cutoff filter of 10,000. Reaction with the fluorescent elastase substrate, bis-(CBZ-Ala-Ala-Ala-Ala)-R110 but not with the trypsin substrate, bis-(CBZ-Ile-Pro-Arg)-R110 confirmed the presence of an elastase. The elastase was inhibited 99% by 10 mM EDTA or 10mM EGTA indicating a requirement for Ca^{++}. Incubation with 10 mM 1,10-phenanthroline resulted in 72% inhibition suggesting that this is a metallo-enzyme. Activity (87%) was retained when heated to 80°C for 10 minutes. These characteristics are similar to those of *P. aeruginosa* elastase or *B. subtilis* thermolysin. The effect of other protease inhibitors, however, suggested that this elastase has unique characteristics. Mtb secreted proteins are believed to play a role in pathogenesis; however the function of only a few of them have been identified. The Mtb elastase identified in culture filtrates may provide a model for studying regulation of expression and export of secreted proteins by this organism.