

## INTERNATIONAL JOURNAL OF LEPROSY and Other Mycobacterial Diseases

OFFICIAL ORGAN OF THE INTERNATIONAL LEPROSY ASSOCIATION

EDITORIAL OFFICE

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VOLUME 62, NUMBER 4

DECEMBER 1994

### EDITORIALS

*Editorial opinions expressed are those of the writers.*

### XIV LEPROSY CONGRESS STATE-OF-THE-ART LECTURES

*We are pleased to have had the opportunity of publishing the full texts of the state-of-the-art lectures presented at the XIV International Leprosy Congress in Orlando, Florida, U.S.A., 29 August–4 September 1993. The first two lectures appeared in the March 1994 issue. The June and September 1994 issues contained additional lectures. The following editorials complete this series of lectures given at the 1993 Congress—RCH*

#### The Microbiology of *Mycobacterium leprae*, Part II. Reflections on Major Developments and Those Responsible for Them\*

Five years ago at the XIII International Leprosy Congress, Dr. Barry Bloom delivered an inspired, visionary state-of-the-art lecture in which he painted a futuristic picture whereby molecular genetics could cure many of our ills, leading, for instance, to a recombinant vaccine against leprosy, and he foresaw how genes from a growing mycobacterium could be inserted into *Mycobacterium leprae* such as to allow its cultivation. Five years later, however, we are now faced with a different reality. We must now begin to redefine our molecular re-

search agenda in light of the conviction on the part of many experts that leprosy can be severely curtailed in the short term solely through aggressive case finding and chemotherapy, and without much input from the extraordinary body of contemporary research on the molecular biology of *M. leprae*.

Let us briefly review progress over the past few years in defining the phenotype of *M. leprae* (what we used to call the bacteriology or microbiology of leprosy) before considering how we should proceed in the future.

Our long-time reasons for studying the immediate and secondary gene products of *M. leprae* are fourfold. We are interested in

\* Based on the state-of-the-art lecture presented at the XIV International Leprosy Congress on 30 August 1993, Orlando, Florida, U.S.A.

studying the interaction of the proteins, carbohydrates and lipids of *M. leprae* with macrophages and T cells in order to understand protective immunity with a view, perhaps, to vaccine development, and also the molecular and cellular basis of immunopathogenesis leading to reactions and nerve damage. Defined antigens also offer the hope of new diagnostic reagents, particularly for epidemiological studies. We also are interested in the physiology and metabolism of *M. leprae* in an effort to understand the non-cultivability of the organism and also the mechanisms by which effective drugs act. In addition, we very much regard *M. leprae* as the paradigm of a successful intracellular parasite and, clearly, a study of it provides clues to the molecular basis of intracellular survival.

Essentially, then, many of the aspects of leprosy that we are all interested in amounts to a study of the gene products of *M. leprae*, namely, proteins, carbohydrates and lipids.

The modern era of the study of the proteins of *M. leprae* began with the pioneering work of Dr. Richard Young, involving the screening of recombinant libraries with monoclonal antibodies. This eventually led to the reproduction of at least eight key immunoreactive proteins: the 70- and 65-kDa proteins, the 36-kDa, two 28-kDa proteins, and some smaller molecular weight products. The cloning and sequencing of many of these proteins ushered in the era of protein homologies, i.e., the extrapolation of sequences to those of proteins from other better studied systems and the consequent conclusions on the functions of these proteins. Good examples of this approach were the discovery of the heat-shock or stress phenomenon, recognized by the likes of Drs. Richard Young, Douglas Young, Shinnick, and Thole, i.e., the observation that many of these initially discovered proteins were probably selectively induced by various stress conditions, probably oxidative stress within the intracellular environment. This same approach gave rise to the recognition of superoxide dismutase by Drs. Colston and Douglas Young, as a key enzyme probably involved in the intracellular life of the bacillus.

This fundamental knowledge on the identification of the key *M. leprae* proteins and their possible roles in the life and reactivity

of *M. leprae* ushered in another great era, the wide-scale availability of these as recombinant proteins, a task ably managed and supervised by the WHO/TDR/IMMLEP Program in its heyday. This has led to enormous progress in defining the roles of these proteins in immunoreactivity (a topic which Dr. Ottenhoff has reviewed), and also in the life of *M. leprae* itself, where many of these proteins are probably involved in protein folding and in the protein export machinery of *M. leprae*.

A very fruitful variation of this early strategy continues in the hands of the likes of Drs. Indira Nath, Josephine Clark-Curtiss, and J. E. R. Thole, involving the screening of cosmid libraries, often with patient sera leading to the discovery of new proteins.

Recent years have witnessed yet newer information and approaches to the definition of structure and function of individual *M. leprae* proteins. One such event is the phenomenon of export proteins. We know from gene sequencing that several true export or secreted proteins are generated by *M. leprae*. However, the one that has captured the most attention is the so-called antigen 85 complex or the  $\alpha$ -antigen. The sequences of this protein complex in *M. leprae* have been established by several investigators, such as Drs. Thole, Clark-Curtiss, Degraeve, and Makino and colleagues in Osaka. However, Drs. Rambukkana and Das in Amsterdam and also Dr. Pessolani at both Colorado State University and Fundação Oswaldo Cruz, Rio de Janeiro, have very effectively shown that the protein is truly excreted by *M. leprae* and is probably one of the most immunoreactive of all *M. leprae* proteins.

There are other recent intriguing aspects of the post-translational modification of *M. leprae* proteins not yet fully understood. Examples are the fascinating splicing and reassembly of the Rec A protein reported by Drs. Davis and Colston and also the concept of true covalently modified proteins, namely, glycoproteins and lipoproteins, as reported by Dr. Douglas Young.

In the meantime, another more direct approach to defining the native proteins of *M. leprae* is now evolving, resulting in new proteins, new principles and understanding. This is the work of three very gifted women

scientists, Dr. Shirley Hunter, Ms. Becky Rivoire, and Dr. Cristina Pessolani, using armadillo-derived *M. leprae* ably produced by Dr. Eleanor Storrs. The approach is theoretically simple but technically extraordinarily demanding, involving the isolation of gram quantities of *M. leprae* from armadillos, fractionation into subcellular fractions (i.e., cell wall, membranes, and cytosol), and the purification of individual proteins from these subcellular fractions in quantities sufficient for sequencing. In other words, the stuff of the aspirations of generations of leprosy biochemists.

Within the cytoplasm/cytosol of *M. leprae*, three major proteins were recognized, major cytosolic protein (MCP)-I, -II, and -III, by SDS-PAGE and purified in sufficient amounts to conduct N-terminal sequencing and Western blotting with the assortment of available monoclonal antibodies from the WHO/TDR/IMMLEP Antibody Bank. MCP-III turned out to be the superoxide dismutase earlier identified by genetic means by Drs. Colston and Young. MCP-II proved to be the 18-kDa protein originally identified by Dr. James Watson and colleagues, among others. However, MP-I, by far the major soluble protein of *M. leprae*, had not been recognized by the initial screening of recombinant banks.

Working intensively with Dr. Vijay Mehra, we were able to purify this protein in considerable quantity (several mg), conduct N-group sequencing on it, cleave it with chemicals and enzymes, and sequence all of the fragments. Dr. Mehra was able to sequence the gene and produce the recombinant protein, allowing Dr. Robert Modlin to examine its immunoreactivity.

The outcome was that the major cytosolic protein, with an apparent mass of 14 kDa, had a true mass of 10.8 kDa and proved to be the *M. leprae* equivalent of the small GroES chaperonin, similar to the *M. tuberculosis* product, but with ten amino acids differences, which results in considerable immunological specificity. And, interestingly, large quantities of it are expressed by *M. leprae* where it represents about 1% of the bacterial mass.

Dr. Pessolani and Ms. Rivoire also were able to isolate the two major membrane proteins (MMP) of *M. leprae*. Membrane-containing fractions of *M. leprae* contain considerable amounts of soluble carbohydrate antigens—lipoarabinomannan (LAM),

lipomannan (LM), and the phosphatidylinositol mannosides (PIMs). By using a detergent biphasic extraction, they were able to separate most of the carbohydrate from the proteins. Dr. Pessolani and Ms. Rivoire are now working with Dr. Warwick Britton and Dr. Nathalie Winter from Sydney on the sequence, expression, and function of MMP-I, a 35-kDa protein.

In the meantime, Dr. Pessolani had developed a protocol for the full purification of the second major protein of *M. leprae* (MMP-II), involving extensive sonication to solubilize it from membranes, Sephacryl gel filtration and C4 reverse-phase chromatography. The availability of this new protein has resulted in some extraordinary insights into the structure and workings of *M. leprae*. Enough of this new protein was generated by using classical biochemical fractionations to allow N-terminal sequencing, to cleave it with proteolytic enzymes to obtain more sequences, to construct oligonucleotides based on peptide sequence, and then to probe the *M. leprae* gene on Southern blots, and, finally, working with Dr. Stuart Cole and Dr. Douglas Smith, to conclude that MMP-II is the *M. leprae* equivalent of a bacterioferritin, since it shared considerable sequence homologies to bacterioferritins from other organisms.

Bacterioferritins are amazing molecules. They contain up to 24 identical subunits forming a spherical shell enclosing a central cavity where up to 4500 ferric atoms may be stored. They are also heme proteins containing protoporphyrin IX as a prosthetic group. To determine whether the new *M. leprae* MCP-II protein is truly a bacterioferritin, Dr. Pessolani re-purified it in its native state by affinity chromatography and was able to recover enough of it to demonstrate a strong Soret band at 408 nm which on reduction shifted to 422 nm and showed the emergence of the  $\alpha$  and  $\beta$  bands, clearly demonstrating the presence of haem. By gel filtration, we estimated the size of the multimer form to be about 380–400 kDa and the sub-unit (monomeric form; the form which we originally identified) to be 18 to 22 kDa, suggesting a multimer of about 20 subunits, close to the bacterioferritins of other organisms. We also were able to estimate the number of iron atoms per multimer, by atomic absorption spectrometry, as somewhere in the 1000–4000 range. Thus, the properties of the new major *M. leprae* pro-

tein are very similar to those of bacterioferritins of other bacterial species.

Dr. Pessolani also has extended this strategy of identifying the full range of key proteins of *M. leprae* expressed *in vivo* (at least in the armadillo). The approach is elegant, straightforward, but again technically demanding, involving one- and two-dimensional gel electrophoresis of various subcellular fractions of *M. leprae*, staining with Coomassie blue, transfer of the Coomassie-blue reactive spots from several such gels onto sequencing membranes, then amino acid sequencing by either Edman chemistry or mass spectrometry, and then the conversion of this information into whole gene sequence relying on Dr. Cole or by first principles. In this way, definitive two-dimensional maps of the array of proteins in the soluble cytoplasm of *M. leprae* were obtained.

The 70-kDa/DnaK and the 65-kDa/GroEL proteins, both highly prominent on such two-dimensional maps, were identified by a combination of sequencing and Western blots. A major spot in the Mr 28-kDa region was excised out and shown to have the correct sequence of the superoxide dismutase. Another spot in the Mr 18-kDa area was excised out and shown to have the published N-terminal sequence of the known 18-kDa protein. The recognition of these known proteins provided excellent internal controls for the veracity of this approach and also served to confirm the physical prominence of these in *M. leprae*. The 10-kDa/GroES was likewise identified. In such two-dimensional maps, stained with silver, it was obvious just how dominant this protein is in *M. leprae*.

From this approach, three additional new proteins were identified by Dr. Pessolani with help from Dr. Cole and his gene sequences. These were described separately at the Congress (C. Pessolani, A. Stanley, P. J. Brennan, Abstract M12); the major one is the *M. leprae* equivalent of the L12 ribosomal protein.

The cell wall of *M. leprae* has also yielded to the same type of molecular definition. We now know an extraordinary amount of information on the cell wall, of a magnitude we could not have dreamed of in The Hague, New Delhi or Mexico City conferences.

Phenolic glycolipid-I (PGL-I), the "external capsule," is still a powerful B-cell immunogen and has its serological practition-

ers despite what the epidemiologists think of it. It is probably anti-bactericidal within the phagocytic cell, since it is known to scavenge oxygen, and perhaps N, radicals. Dr. Schlesinger has published intriguing evidence that PGL-I on the surface of *M. leprae* binds to complement 3b which, in turn, binds to complement receptors 1, 3 and 4 on mononuclear phagocytes, and that this mechanism is responsible, in part, for phagocytosis of *M. leprae*.

However, it is in the definition of another cell wall product of *M. leprae*, lipoarabinomannan (LAM), that most recent progress has been made. This work is largely the accomplishment of another gifted colleague, Dr. Delphi Chatterjee.

Dr. Chatterjee has been very successful in isolating the major soluble carbohydrate antigens of *M. leprae* and actually resolving them on SDS-PAGE gels. Intriguingly, they are all structurally related. They are the phosphatidylinositol mannosides (PIMs), the lipoarabinomannans (LAMs), and the lipomannans (LMs). She also has evidence that these are excreted by *M. leprae*. They also all have important biological properties relevant to leprosy as a disease.

The major PIM of *M. leprae* contains six mannose residues in all, and is thus a PIM<sub>6</sub>, a phosphatidylinositol hexamannoside. LM is an extension of this and contains 20–21 mannose residues in all. On the other hand, LAM has an arabinan attachment, and now recently we have shown that about 40% of the termini of the arabinan are capped with mannose residue somewhat similar to the mannose-capped LAM (ManLAM) of *M. tuberculosis*.

A considerable body of evidence, some of it from Dr. James Krahenbuhl, some of it from Dr. Gilla Kaplan, some work from Drs. Bloom and Chan, and Dr. Chatterjee and colleagues, is now beginning to implicate LAM in several immunosuppressive events associated with leprosy and, hence, some people have called it the first *M. leprae* virulence factor. It is a major B-cell antigen, but it also suppresses generalized lymphocyte proliferation,  $\gamma$ -IFN activation of macrophages, and protein-antigen presentation and processing. It apparently aids in the intracellular replication of *M. leprae* by neutralizing oxygen radicals and, above all, it now seems to be involved in the stimulation of "bad" cytokines such as  $\alpha$ -TNF.

There have been two recent major de-

velopments in our understanding of the physical nature of mycobacterial cell walls. Dr. Hiroshi Nikaïdo has studied the physical organization of the mycolic acids using X-ray diffraction, and concluded that they are perpendicular to the cell wall surface and arranged in a fashion to produce an asymmetric bilayer structure, a pseudo outer membrane. Secondly, studying cultivable mycobacteria, Drs. Trias and Benz have concluded that there are true porins in this pseudo outer membrane. However, they are few and far between.

So, now, at our XIV International Leprosy Congress of 1993, we have a thorough fundamental knowledge of the nature of the pathogen. We have identified most of the cystolic proteins (the 10-kDa GroES, the 65-kDa, the superoxide dismutase, the 18-kDa, the L-12 ribosome protein). We have identified the major membrane proteins (the new bacterioferritin, the 35-kDa). From the work of Dr. Philip Draper, we have a good knowledge of the nature of peptidoglycan (especially the presence of glycine). From the work of Drs. McNeil and Daffe, we know of the new "linker arm" which is made up of two sugars and a phosphodiester which is responsible for the attachment of the rest of the cell wall (i.e., the mycolyl-arabino-galactan) to peptidoglycan. The galactan component apparently exists as  $\alpha$ -helical coils of linear 1  $\rightarrow$  5- and 1  $\rightarrow$  6-linked galactofuranose residues. The structure of arabinan has been established as has also the method in which mycolic acids are attached to it in a truly amazing structural unit, a tetramycolylpentaarabinofuranoside, to form the bottom half of an asymmetric bilayer complemented with PGL-I and which may have occasional porins. And we know much about the structure of LAM and its role in the pathogenesis of leprosy.

"Four things come not back: the spoken word; the sped arrow; time past; the neglected opportunity" (Omar ibn Al-Halif; Aphorism)

In response to those immortal words, I can say on behalf of all molecular biologists that we did not waste the opportunity given to us; we have produced a comprehensive, thorough, elegant definition of the leprosy bacillus.

In deciding how we should proceed, I am reminded of the words of St. Jerome ("The scars of others teach us caution" in Letter 54), which we can paraphrase, "The scars of tuberculosis teach us caution." Despite the present hopeful climate of global leprosy, we must maintain a strong fundamental microbiological research program free of the vicissitudes of global predictions and World Health Assembly promulgations. And it should be based on helping in leprosy control efforts, more in understanding pathogenesis and developing tests for measuring disease incidence and less on vaccine development, and also based on the fundamental scientific worth of the study of such a successful obligate intracellular parasite that has caused and continues to cause such hardship.

"It had always seemed to the doctor unfair that leprosy did not preclude all other diseases (leprosy was enough for one human being to bear)." Graham Green in *A Burnt-Out Case* (1960)

I would like to thank my colleagues in Colorado, especially Ms. Rivoire, Drs. Pesolani, Hunter, Chatterjee and Chitale, and my colleagues at Albert Einstein College of Medicine in New York (Drs. Bloom and Mehra), the Leonard Wood Laboratories in Cebu (Drs. Walsh, Cellona, Fajardo and Abalos), Dr. Modlin at the University of California, Los Angeles, and my friends from WHO (Drs. Noordeen, Engers, Grosset and Ji) for helping me to think globally when leprosy research was in its prime.

"In dealing with communicable disease, the closer a program gets to eliminating a disease, the more likely it is that the program gets eliminated rather than the disease." Donald Kopenoff, Associate Director, Division of Tuberculosis Elimination, Centers for Disease Control

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*A full list of references to published papers that underlie this presentation is available from Dr. Brennan.*