

TB control is only possible through the integrated action of multiple factors at the social and biological level



**Complexity – Franklin Sotolongo**  
Oil/canvas

'Sometimes the page breathes:  
swarms of symbols and meaning,  
bound and disperse in magnetic rotation  
...'

***A Draft of Shadows (Poem) – Octavio Paz***

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Franklin Sotolongo qualified as a medical doctor from the Faculty of Medicine, Cuba. His specialization in Microbiology was obtained at the Medical Sciences Institute, Cuba. He did a post-graduate fellowship in Neisseria, France, again specializing in Microbiology. He is an assistant professor of Microbiology. He is an expert in clinical meningococcal vaccine assessment and in developing bactericidal assay for antibody response evaluation. He is working on immunogenicity evaluation of meningococcal vaccine in several clinical studies in different countries, Phase I–II (WHO-sponsored). He also serves as PAHO–WHO temporary advisory for meningococcal disease and meningococcal vaccine evaluation, and follow up (Phase IV). Sotolongo has authored and co-authored several publications on Neisseria meningitis and meningococcal vaccines. He is also co-author of the Cuban meningococcal B vaccine patent, and author of a new culture medium for Neisseria meningitis patent.

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## CHAPTER 7

# Antibody Responses in Tuberculosis

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Studies on the antibody response to MTB infection date back to 1898 when Arloing documented specific antibody production in TB patients (1). Since then, research has been directed towards identifying antigens having serodiagnostic potential (2) and understanding the role of antibodies in disease pathogenesis and protective immunity (3). In this chapter, we focus on antibody responses associated with the stage of infection, with an emphasis on IgG responses to MTB protein antigens. Other aspects of the antibody response, such as its relationship to the pathogen life cycle and response to non-protein antigens of MTB, have been recently reviewed (4). The chapter is organized in three sections. The first section describes antibody responses to MTB during various stages of infection and disease. The second section explores possible causes for the heterogeneity in the antibody response to MTB. The third section describes novel immunoassay tools that have entered the study of TB serology.

## Antibody Response and Natural History of Infection

MTB infection is usually acquired through inhalation of airborne droplets containing the bacterium. Engulfment of tubercle bacilli by antigen presenting cells, such as dendritic cells and macrophages, initiates immunological events that culminate in the formation of the tuberculous granuloma. The granulomatous

response, albeit sufficient to arrest bacterial growth and contain infection, is incapable of eradicating it. Immune containment leads to an asymptomatic state called latent infection that may be lifelong. Compromise of the immune response results in resumed bacterial multiplication and reactivation of the disease. Immunocompetent individuals infected with MTB have a lifetime reactivation risk of 10 per cent, but for immunocompromised individuals (e.g. coinfection with HIV) risk can be as high as 10 per cent per year (5). Below we describe the antibody response to MTB during various stages of infection.

### **Primary Progressive Tuberculosis**

When host immunity fails to contain primary infection with MTB, primary progressive TB ensues. Little is known about the antibody response during this manifestation of disease. In children under two years of age, where primary TB is most commonly observed (6), levels of specific antibodies are low (7) due to the paucibacillary nature of the disease and the associated low antigen burden, to the immaturity of the child's immune system, or to both. Moreover, several factors may confound the antibody response to natural infection in this age group. One, for example, is the presence of maternal antibodies; another is vaccination with BCG, which is given at birth in most countries having high TB endemicity (8).

### **Latent Infection**

During latent infection, a small number of tubercle bacilli presumably persist inside granulomas where bacillary multiplication is restricted by the host immune response. During this state, some bacterial proteins may induce measurable levels of antibody. These antigens include secreted virulence factors (e.g. ESAT-6 and CFP-10) and components of the bacterial stress response (e.g. alpha-crystallin). Repeated exposure (reinfection) to MTB may also play a role in the specific antibody response in asymptomatic individuals. For example, a higher proportion of antibody responses to RD1 region antigens has been seen in healthy, latently infected subjects from TB endemic regions as compared with individuals who live in areas of low TB incidence. Moreover, antibody responses are higher in contacts of active TB patients than in community controls (9, 10). (The RD1 region is a fragment of the mycobacterial genome found in virulent tubercle bacilli but deleted from BCG vaccine strains) (11). Furthermore, antibody to alpha-crystallin is associated with contact with smear-positive TB patients or heavy occupational exposure to MTB, and it is present in individuals with self-healed TB (a condition similar to latent infection but presumably associated with higher bacillary numbers) (12). Particularly intriguing is the possibility that antibodies to some antigens may be associated with increased risk of reactivation disease. Antibodies to ESAT-6 have been

found in individuals who are at risk of reactivating disease, for example, individuals reporting past (inactive) TB, history of close contact with infectious cases, or recent immigration from a TB-endemic area (13). Why increased risk of reactivation may be accompanied by increased antibody to this antigen is uncertain, but it may reflect increased bacillary burden higher than typically associated with stable latent infection (see subsection: Incipient Disease). It has also been proposed that increased interferon gamma (IFN $\gamma$ ) response by T cells to ESAT-6 is associated with a risk of reactivation (14).

### **Incipient Disease**

As incipient (or preclinical) disease we refer to a stage of asymptomatic infection preceding onset of clinical manifestations of reactivation disease (14). In this situation, some bacterial proteins may induce antibody responses before clinical manifestations appear due to relative immunodominance and/or increased production. Since reactivation disease is a relatively rare event, exploring immune responses during preclinical TB would require extended longitudinal studies involving large numbers of subjects. Consequently, the investigation of the antibody response associated with preclinical TB is usually limited to conditions characterized by greater reactivation risk, such as HIV coinfection. Studies with HIV-infected, TST-positive individuals have shown that specific antibodies appear in serum as early as two years prior to clinical diagnosis of TB (15, 16). These include antibodies to malate synthase, alpha-crystallin, ESAT-6, and CFP-10 (16).

Preferential early recognition of particular bacterial antigens may have several, non-mutually exclusive causes. For example, antigen burden may increase due to overall increased numbers of multiplying bacilli, increased production of particular antigens by multiplying bacilli (17), and by increased levels of free antigen (14). Work with animal models supports increased antibody production with progression to disease (18). Under these circumstances, it is conceivable that detection of antibodies against proteins that have a low threshold for antibody production occurs prior to the clinical manifestations of disease.

### **Active Disease**

Due to the chronic nature of MTB infection, neither a transition from IgM to IgG antibodies nor a sharp rise in antibody level is seen as latent infection progresses to active disease. Instead, reactivation tends to be associated with a gradual increase in serum levels of IgG antibody, which is detected in at least 90 per cent of reactivation disease patients (19). Moreover, proteins targeted by antibodies vary greatly across patients (19), and both host and pathogen factors may contribute to this variation (see section: Heterogeneity in Antibody Response). Nonetheless, recognizable antibody patterns have

been described. For example, antibodies to the 38-kDa antigen are associated with high bacillary burden, as seen in smear-positive and advanced TB (20). In contrast, antibodies to the 19-kDa lipoprotein antigen are associated with paucibacillary disease, which correlates inversely with recognition of the 38-kDa antigen (21). Moreover, it has been suggested that antibodies to antigens such as alpha-crystallin and ESAT-6 tend to be absent in patients that are sero-positive to the 38-kDa antigen (13). None of these antibodies, however, is considered robust enough to constitute, by itself, a marker of disease.

## **Extrapulmonary Tuberculosis**

Extrapulmonary tuberculosis, which may involve pleura, meninges, pericardium, and, most commonly, lymph nodes (22), is seen mainly in children and in HIV-infected individuals (23). At least two factors may make the antibody response particularly difficult to assess in extrapulmonary TB. One is that antibody levels tend to be lower in extrapulmonary TB than in pulmonary TB. Another is that the antibody response seen in extrapulmonary TB may vary with the organ affected. For example, expression profiles of some PE/PPE proteins have been reported as being organ-specific (24), presumably because bacterial gene expression varies in response to different micro-environments. Moreover, antigen accessibility to the host immune system may vary in different tissues. When detected, antibody responses in extrapulmonary TB show varied patterns among individuals (similar to that seen in pulmonary disease), even when the same organs are affected. In some forms of extrapulmonary TB, specific antibodies can also be detected in local fluids, such as the pleural fluid in patients having tuberculous, pleural effusion, and cerebrospinal fluid in tuberculous meningitis patients (25, 26).

## **Coinfection with HIV**

HIV-infected individuals are at increased risk of reactivation of TB. Subjects having asymptomatic HIV infection retain the ability to generate high-affinity antibodies (15) and humoral immunity to recall antigens (27). Indeed, MTB-specific antibodies are detected in HIV-infected individuals with active TB (28, 29), but at lower levels than HIV-negative persons (28). Moreover, changes in antibody responses associated with incipient disease have been described in HIV-infected persons, as reported in subsection: Incipient Disease. A pathology recently described with HIV and MTB coinfection is the immune reconstitution inflammatory syndrome (also known as immune reconstitution disease) associated with highly active antiretroviral treatment. In this syndrome, previously subclinical or partly treated opportunistic infections clinically deteriorate during antiviral treatment due to rapid immune restoration and lack of compensating immunoregulatory mechanisms. Mycobacterial infections are

most frequently implicated in immune reconstitution inflammatory syndrome (30). The antibody response to TB in immune reconstitution disease has not been fully explored, but a lower risk of developing TB was found to be associated with antibodies to the bacterial lipid PGL-Tb1 (31). Unlike the response to protein antigens, the antibody response to glycolipids, which utilizes the CD-1 restricted lipid antigen presentation pathway, may be preserved and readily measurable in HIV-positive individuals.

## Heterogeneity in Antibody Response

Variation is a common feature of biological systems. Both populations and individuals within a species vary. Among infectious diseases, TB constitutes a prime example of the effects of intra- and inter-population variation, because the outcome of exposure of the human host to MTB is determined by interactions ranging from the molecular to the population level (32), with variation operating at each of these levels. For example, it is estimated that only 30 per cent of exposed persons show evidence of infection (32), and, of those infected, only 10 per cent ever become ill. Within the latter group, disease occurs with varying time of progression, clinical presentation, and severity. Both humoral and cellular immune responses to MTB echo this variation. As alluded to in previous sections, the targets and the magnitude of the immune response to MTB show marked variation among individuals (19, 33, 34), with both host and pathogen factors being implicated (32, 35, 36).

## Pathogen Factors

The physiological state of MTB depends upon the micro-environment it experiences. Morphology and distribution of acid-fast bacilli vary in the tuberculous lung (37). Tubercle bacilli found in the central area of granulomas are mostly extracellular, whereas those in the pericavitary area predominantly exist inside macrophages (38). Accordingly, bacteria inside granulomas show different mRNA expression profiles than those in the pericavitary areas or in the distant lung (38). Recent studies have shown that lipid body-laden, persister-like bacilli can represent up to 86 per cent (average 45 per cent, minimum 3 per cent) of bacilli in the sputum of active TB patients (39). Furthermore, latently infected individuals may harbour replicating bacteria, as indicated by the ability of isoniazid, which is most active against replicating bacilli, to reduce risk of reactivation up to 90 per cent in children (40). Thus, little doubt exists that granulomas are dynamic structures (41). If tubercle bacilli in different physiological states express different immunodominant antigens, as suggested

(17, 42), antigenic diversity would ultimately impact the antibody repertoire expressed by different patients.

When comparing responses in TB patients across geographical areas, an additional source of variation may be constituted by the antigen composition of the infecting strain. Different clinical isolates differ with regard to expression of antigens such as ESAT-6, Antigen 85, and the 19kDa antigen (43, 44). Strain-to-strain variation has also been reported for the family of PE/PPE proteins, which are characterized by the presence of Pro-Glu/Pro-Pro-Glu motifs (45–48), suggesting that PE/PPE proteins may provide tubercle bacilli with a dynamic antigenic profile responsive to micro-environment changes (49). A different view, however, was expressed by investigators reporting limited genetic diversity among genes encoding antigenic targets, such as members of the PE and PPE family of proteins (50). In that view, key MTB antigenic proteins have negligible structural variation worldwide, with the immune system exerting limited selective pressure on genes coding for antigens (50). Other authors have emphasized adaptation of infecting strains to specific host populations in geographically defined areas with an ensuing specific ‘compatibility’ between particular infecting strains and human populations (51). How these processes affect the bacterial targets of the immune response remains to be elucidated.

## Host Factors

The class of ‘active disease’ includes individuals at various stages of disease progression and severity. For example, pulmonary TB can be paucibacillary (low antigen load) or multibacillary (high antigen load), with overall antibody levels being affected by antigen load. Moreover, previous exposure to MTB antigens may contribute to variation in the antibody response. For example, the antibody repertoire in reactivation disease may differ from that of progressive, primary TB because in the former case the host immune system has been chronically exposed to bacterial antigen. It has been shown that, in latently infected individuals, repeated exposure to MTB may cause increased levels of antibodies to certain antigens (9, 10). In addition, a memory response resulting from past disease can affect the kinetics of antibody response in reinfection disease. In one example, pre-existing antibodies to *M. bovis* antigens in calves were proposed to interfere with the expression of antigen in a subsequent *M. bovis* infection (52). Antigen accessibility (e.g. in extrapulmonary TB) might also affect the antibody repertoire seen in active TB. Moreover, various aspects of immune dysregulation may ultimately determine the antigens expressed by the pathogen or recognized by humoral immunity. Little evidence is currently available to support any of these scenarios.

Since host genetics play a role in innate and adaptive immune responses to TB (53, 54), the antibody response to particular antigens may also be

influenced by specific host genetic traits. An example is the association between high levels of antibodies to 38kDa antigen and HLA-DR15, a subset of HLA DR2 that is also independently associated with smear-positive pulmonary disease (55–57). Additional examples of the association between MHC genes and the antibody repertoire to mycobacterial antigens derive from murine studies (58). The relationship between host immunogenetics and antibody profiles in TB patients, which has been investigated only sporadically (21, 55–57), has been recently reviewed (4). It is worth noting that preferential transmission of specific strains among distinct ethnic groups may also occur (59), according to the concept of host–pathogen compatibility presented in the subsection: Pathogen Factors. Thus, since antigen expression levels vary among strains, antibody profiles may be affected by host ethnicity not only because of host genetic traits, but also due to the antigen composition of the strains preferentially harboured.

## Novel Immunoassay Platforms

As mentioned in the previous sections, antibodies to MTB antigens can be detected at various stages of infection and disease, but a systematic understanding of the response remains to be developed. In the era of genome-scale investigations, it is necessary to apply new tools that make it possible to interrogate the entire proteome of MTB to identify stage-specific antibody responses and define changes in antibody responses occurring during progression from infection to disease. The following section provides a brief description of novel immunoassay techniques that utilize high-throughput approaches and multiplexing, such as protein microarray and fluorescent bead technology.

### Protein Microarrays

Protein microarrays, also known as protein chips, are miniaturized, parallel assay systems that contain small quantities of purified or non-purified proteins, synthetic peptides or protein fragments that are immobilized onto modified glass surfaces. This system allows simultaneous screening of interactions in a single experiment utilizing a variety of analytes in small volumes (60). From printing methods to statistical analyses, protein microarrays have adapted techniques originally developed for nucleic acid microarrays (61–63). However, the protein microarray technology has unique characteristics related to the variable physico-chemical properties of individual proteins (64). Protein chips typically contain only a fraction of the proteome, such as a particular set or family of proteins (42, 65, 66) or small viral proteomes (67). However, recently developed techniques make it possible to synthesize and

probe the entire proteome of micro-organisms of known genome sequence (60, 68, 69).

Proteins have diverse binding chemistries; thus, finding a common surface suitable for different proteins is challenging. An ideal surface should allow optimal binding of proteins to the chip surface with minimal non-specific binding of the probe to the chip surface or to negative controls. To this end, slide-surface chemistries have been adapted from the western blot method or from nucleic acid microarrays. Binding is achieved either by covalent linkage between solid phase and protein (e.g. via epoxy groups) or by non-covalent interactions (with nitrocellulose-coating or nickel-affinity-tag binding) (63). Occasionally, a linker RNA or protein molecule on the slide surface binds to the desired protein (70). A three-dimensional hydrogel with reactive groups that can bind to proteins or peptides can preserve the three-dimensional structure of proteins on the array (71). The surface chemistry is important, because preparation of the proteins, density of printing, and functionality of printed proteins vary depending upon the selected surface. Another important feature of protein microarrays is the mode of arraying. Robotic printing can be of two types: contact and non-contact printing. In contact printing, a pin is first immersed into a protein solution and then it touches the slide surface to create a spot. Pins can be solid or slit; the latter type contains a reservoir and has the advantage of applying multiple spots after a single immersion. Non-contact arraying uses ink-jet or piezoelectric printers for spotting on slide surfaces. Piezoelectric printers use voltage to quantitatively control droplet formation at the end of a capillary.

Generation of a proteome chip carrying all the proteins of a micro-organism has the potential of comprehensive and unbiased screening, but it requires the implementation of efficient, high-throughput methods. In principle, any organism having a complete genome sequence is a candidate for proteome microarray fabrication. Advances in molecular biology have allowed faster production of libraries of open reading frames cloned in suitable expression vectors, which is the first step in the generation of protein microarrays (72, 73). High-throughput protein purification has typically been the next step towards realization of proteome chips. Chips carrying purified proteins of *Saccharomyces cerevisiae* and *Escherichia coli* have been developed to study protein-protein interactions, protein glycosylation, and antibody specificity (60, 68, 74, 75). A need for protein purification has been circumvented by printing unpurified proteins or whole cell lysates (69, 76). In a technique called NAPPA (nucleic acid programmable protein arrays), proteins are expressed in situ using a cDNA spotted array that is bathed with the cell-free in vitro transcription/translation mixture (77). Expressed proteins are immobilized to the slide surface by affinity capture.

Proteome chip technology has entered the field of TB research. In one study, protein microarrays were obtained by printing on nitrocellulose-coated glass slides 960 fractions from cytosol and culture filtrate from MTB cultures, and probing the resulting protein microarray with sera from TB patients and controls (76). In a second study, microarrays of overlapping synthetic peptides were constructed for several known MTB antigens and shown to effectively help detect antibody responses to those antigens (78). In a third study, a full proteome chip (> 90 per cent of the MTB proteome) was developed by a published method, (69) which involves use of high-throughput cloning in an *E. coli* expression vector, expression of recombinant protein in vitro transcription/translation, and direct printing of expression mixes onto nitrocellulose-coated glass slides without purification. The resulting proteome microarray chip makes it possible to interrogate the entire MTB proteome for recognition by specific antibody (Molina et al., unpublished data) and to detect antibody markers of active disease (Kunnath et al., unpublished data).

Both microarray approaches utilizing whole proteins do not allow for accurate quantification of MTB protein per spot. Use of protein fractions of MTB lysates has the advantage of utilizing proteins in their native configuration (as opposed to recombinant proteins expressed in *E. coli*), but it is not amenable to full-proteome coverage. Moreover, it requires downstream methods, such as mass spectrometry, for identification of reactive species. Peptide arrays allow rigorous calibration of protein amount per spot and make it possible to map epitopes. However, due to potentially extremely high complexity of data (132,000 peptides (10 mer) are needed to cover 4,000 proteins having an average gene length of 1kb) and the cost of peptide synthesis, they are better suited for in-depth analysis of selected seroreactive antigens than for full proteome coverage.

## Microsphere Arrays

Bead-based assay systems use polystyrene microspheres as solid support rather than the glass surface of planar microarrays. These microspheres are internally dyed with two spectrally distinct fluorochromes and generate specific spectral signatures (79). A third fluorochrome coupled to a reporter molecule is used to detect antigen-antibody binding on the microsphere surface. The assay uses flow cytometry to quantify the amount of captured target on each individual bead. Bead-based systems have several attractive features (e.g. increased surface area for binding reactions and no requirement for washing steps), and they allow multiplexing (up to 100 analytes simultaneously) (80). However, bead-based systems require purified protein and therefore are not designed for full-proteome coverage. Use of this technology in TB research has been pioneered in a study on the antibody response to selected antigens of MTB in experimentally infected monkeys (81).

## Conclusion

The chronic nature of the infection with MTB and its high endemicity in many regions of the world give rise to a complex epidemiological pattern in which exposure, latent infection, active disease (exhibiting various degrees of severity), re-exposure, and past disease are conditions that can be simultaneously present in the population and even in the same individual at different times. Such patterns, together with particular genetic traits of the infecting strain and infected host, affect the immune response. One of the consequences is the variation of antigenic targets from one individual to another. We anticipate that comprehensive interrogation of the antibody response with genome-wide methods will make it possible to decipher the response in relation to the stage of infection and disease, clinical picture, and outcome. Some of these methods have been reviewed in the present chapter; for other techniques that use label-free detection and non-fluorescent labels, the reader is directed to earlier publications (82–87). A thorough understanding of the antibody response will make it possible to determine whether antibody responses are robust enough to serve as diagnostic and prognostic markers of TB.

### Box 7.1

#### **Antibody response and natural history of infection**

##### *Primary progressive tuberculosis*

- No pre-existing antibody responses
- Possible cross-reacting antibodies due to BCG vaccination and exposure to non-pathogenic mycobacteria

##### *Latent infection*

- Possible transient antibody response during early infection
- Chronic response characterized by low/undetectable antibody levels
- Potential recognition of bacterial antigens expressed during dormancy
- Increased antibody levels in high-exposure settings
- Increased antibody responses may indicate progression to disease

##### *Incipient disease*

- Precedes onset of clinical manifestations and microbiological evidence of disease
- Antibodies appear up to 2 years prior to clinical manifestations

##### *Active disease*

- Antibody responses seen in 90 per cent of patients
- Gradual increase in specific IgG levels
- Increased repertoire
- Potential effect of pre-existing antibody response

**Box 7.2****Antibody responses during specific conditions***Extrapulmonary tuberculosis*

- Antibody repertoire depends upon the organ involved
- Presence of antibodies in pleural and cerebrospinal fluids
- Low antibody levels due to low burden and/or poor antigen accessibility

*HIV infection*

- Lower antibody levels than in HIV-negative individuals
- Lipid antigens are presented by CD-1 restricted pathway
- Antibody responses during immune reconstitution syndrome to be elucidated

*Treatment*

- Increase in antibody repertoire and level with no change in affinity
- Biphasic response of antibodies to some protein antigens
- Possible restoration during relapse

*Vaccination*

- Possible transient antibody response

**Box 7.3****Factors contributing to the variation in antibody responses during disease***Pathogen factors*

- Micro-environment
- Growth state of bacteria
- Strain variation

*Host factors*

- Stage/severity of disease
- Presence of cross-reacting antibodies
- Previous exposure to antigen
- Host genetics

**Box 7.4****Novel immunoassay platforms***Platforms using labels for detection*

- Fluorescence
  - Planar Microarrays
  - Suspension arrays (microbeads)
- Magnetic beads

*Platforms with label-free detection*

- Optical sensors
- Electrical sensors
- Acoustic sensors
- Mechanical Sensors

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