NOTES

Antigens of *Mycobacterium tuberculosis* Recognized by Antibodies during Incipient, Subclinical Tuberculosis

Krishna K. Singh,1 Yuxin Dong,1 John T. Belisle,2 Jeffrey Harder,2 Vijay K. Arora,3 and Suman Laal1,4*

Department of Pathology, New York University School of Medicine,1 and New York Harbor Health Care System,4
New York, New York; Department of Microbiology, Immunology and Pathology, Mycobacteria Research Laboratories, Colorado State University, Fort Collins, Colorado;5 and Lala Ram Sarup Institute of Tuberculosis and Respiratory Diseases, New Delhi, India6

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Serum samples obtained from human immunodeficiency virus (HIV)-infected tuberculosis (TB) patients months prior to clinical TB were used to delineate the profile of *Mycobacterium tuberculosis* culture filtrate proteins recognized during subclinical TB. A subset of ~12 antigens was recognized by antibodies in these serum samples. Antibodies to two of these antigens (81 [88]-kDa malate synthase [GlcB] and MPT51) were present in serum samples obtained during incipient subclinical TB in 19 (~90%) of the 21 HIV-infected TB patients tested. These antigens will be useful for devising diagnostic tests that can identify HIV-positive individuals who are at a high risk for developing clinical TB.

Our earlier studies of antibody responses to *Mycobacterium tuberculosis* antigens at different stages of tuberculosis (TB) (latent TB, noncavitary TB, cavitary TB) and in different classes of TB patients (non-human immunodeficiency virus [HIV] infected or HIV infected) provided evidence that the profile of antigens recognized by antibodies is altered with disease progression (10–12, 15–17, 19). We delineated a subset of ~12 culture filtrate proteins (CFPs) that is recognized by HIV− TB+ and HIV+ TB+ patients with noncavitary TB but not by healthy, purified protein derivative-positive (PPD+) individuals (15). In contrast, HIV− TB+ patients with extensive cavitary lesions have antibodies against the same ~12 antigens and also an additional subset of ~10 CFPs (15). Two of these ~12 antigens are the MPT51 (Rv3803c) and 81-kDa malate synthase (GlcB) protein (Rv1837c; same as the 81 [88]-kDa protein reported earlier) (11, 12, 15). The immunodominance of the 81 (88)-kDa protein in HIV− TB+ and HIV+ TB+ patients has been reported in cohorts from the United States, India, Uganda, South Africa, and Brazil (7, 14, 17).

*M. tuberculosis* is a slow-growing organism, and it takes several weeks to months for an infection to progress to clinical TB. Insight into the antigens expressed in vivo during subclinical TB could contribute to our understanding of the host-pathogen interaction that leads to progression to clinical TB. Our earlier studies provided evidence that antibodies to some CFPs of *M. tuberculosis* are present in retrospective serum samples obtained from HIV+ TB+ patients during the months prior to manifestation of clinical TB (subclinical TB), and one-dimensional (1D) Western blot assays of semipurified CFP fractions indicated that the 81 (88)-kDa protein was one of the antigens recognized (11). Since antibodies are markers of antigens expressed in vivo, Western blotting of 2D fractionated CFPs with subclinical TB serum samples has been performed to delineate additional antigens expressed during subclinical TB. Antibodies in the subclinical TB serum pool identified the same subset of ~12 CFPs recognized by HIV− TB+ and HIV+ TB+ noncavitary TB patients, including the 81 (88)-kDa and MPT51 proteins. Antibodies to the purified 81 (88)-kDa and/or MPT51 proteins were detected in the retrospective serum samples from 90% of the HIV+ TB+ patients. Since healthy PPD+ individuals lack antibodies to these proteins, the presence of antibodies to these antigens in the subclinical TB serum samples suggests that in vivo *M. tuberculosis* replication may begin months before progression to clinical TB occurs.

Serum samples were obtained from 21 *M. tuberculosis* culture-positive HIV+ TB+ patients attending the infectious diseases clinic at the Veterans Administration Medical Center, New York, N.Y., during the 1980s and 1990s (before antiretroviral drugs became available) who were being monitored for progression of HIV infection and developed TB during follow-up; serum samples obtained from these patients 3 months to 2 years prior to development of TB are HIV+ subclinical TB serum samples (11). Three HIV+ patients presented with TB. Serum samples were also obtained from 20 HIV+ TB− individuals on antiretroviral therapy. Serum samples from 29 healthy PPD+ (>10-mm induration) and 10 healthy PPD− individuals were used as negative controls; 22 of the 29 PPD+ individuals and 3 of the 10 PPD− individuals were recent immigrants from countries where TB is endemic (India, China, Cameroon). Serum samples from 40 untreated HIV− TB−
patients (acid-fast bacillus [AFB] smear-positive, cavitary TB) from India were also tested.

Enumeration of different lymphocyte subsets was done by standard procedures with the Simultest CD3-CD4 and CD3-CD8 reagents (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) (11). Flow cytometry was carried out with a FACScan apparatus (Becton Dickinson).

To determine the profile of CFPs of \textit{M. tuberculosis} recognized by antibodies in the subclinical TB serum samples, the lipoarabinomannan-depleted CFP preparation (\textit{M. tuberculosis} H37Rv; ATCC 27294) was used for all of our earlier studies was used (12, 15–18, 20). This preparation contains >100 proteins, and the 2D protein profile and the repertoires of antigens recognized at different stages of TB and in different classes of TB patients have been described previously (15, 16, 20). 2D Western blot assays of CFPs were probed with a pool of six serum samples collected during subclinical TB from HIV+ TB patients or PPDb controls (1:200) (15, 16). The serum pools were preabsorbed against \textit{Escherichia coli} lysates to reduce levels of cross-reactive antibodies (12, 15, 16).

Healthy PPDb+ individuals have been shown to possess antibodies directed against approximately two to four proteins of \(~30 \text{ to } 32\) kDa, three of which could be identified as Ag85A, -B and -C and approximately one to three proteins of \(~65\) kDa, one of which was glutamine synthase (15, 16, 20). The serum pool from PPDb+ individuals in this study recognized five proteins at \(~30 \text{ to } 32\) kDa, three of which are Ag85A, -B, and -C, and one protein of \(~65\) kDa (Fig. 1A). The subclinical TB serum pool had antibodies against the above six proteins and also \(~12\) additional CFPs (Fig. 1B). The profile of antigens recognized by the subclinical TB serum pool is identical to the profile recognized by antibodies in HIV+ TB+ and HIV+ TB+ noncavitary TB patients and includes the 81 (88)-kDa and MPT51 proteins (15).

Cloning and expression of the 81 (88)-kDa protein (Rv1837c) and purification of the recombinant protein have already been described (18). The MPT51 protein (Rv3803c) was expressed without the signal peptide (33 amino acids). The gene was PCR amplified from the \textit{M. tuberculosis} H37Rv genomic DNA with forward (5'-CCGCCCATATGGCCCCCACA TACGAGAA-3') and reverse (5'-CACCTAAGCTTGCGGA TCGCACCAGA-3') primers containing NdeI and HindIII sites (underlined), respectively; cloned into pET-23b (+) (Novagen, EMD Biosciences Inc., Madison, Wis.); and sequenced to verify the reading frame, and the recombinant plasmid was transformed into \textit{E. coli} BL21(DE3)/pLysS. A single colony of \textit{E. coli} BL21(DE3)/pLysS/pET-23b (+)-MPT51 was used as a source of recombinant MPT51 whose identity was confirmed by reactivity with anti-His and anti-MPT51 (IT52) monoclonal antibodies. The recombinant MPT51 protein was purified by nickel affinity chromatography in accordance with the manufacturer’s (QIAGEN Inc., Valencia, Calif.) protocol.

The presence of antibodies to the purified 81 (88)-kDa and MPT51 proteins in individual patients was determined by enzyme-linked immunosorbent assay. The antibodies were coated at 4 µg/ml (50 µl/well), and the patient or control serum samples were tested at 1:50 for the 81 (88)-kDa protein or at 1:25 for the MPT51 protein. A mixture of alkaline phosphatase-conjugated protein A (1,200; Sigma, St. Louis, Mo.) and anti-immunoglobulin A (1,100, Sigma) was used to detect the antigen-bound antibodies. The mean optical density (OD, 490 nm) of the healthy PPDb+ and PPDb- individuals in the different enzyme-linked immunosorbent assays ranged from 0.17 to 0.36 for the 81 (88)-kDa protein and from 0.22 to 0.37 for the MPT51 protein. In each experiment, the mean OD of this group plus 3 standard deviations was used to determine the cutoff (range, 0.40 to 0.67 for the 81 [88]-kDa protein and 0.51 to 0.72 for the MPT51 protein) to identify positive patients. The OD values for the positive patients ranged from above the cutoff to as high as 3.5 for the 81 (88)-kDa protein and 3.3 for the MPT51 protein. Each specimen was tested two to four times, and only patients who were consistently positive or positive two of three or three of four times were considered positive.

None of the healthy PPDb+ or PPDb- individuals, whether from countries where TB is endemic or the United States, had anti-81 (88)-kDa or anti-MPT51 protein antibodies (Fig. 1C). In contrast, anti-81 (88)-kDa protein antibodies were present in 28 (70%) and anti-MPT51 antibodies were present in 24 (60%) of the 40 HIV+ TB+ patients; together, the two antigens identified 33 (83%) of the 40 HIV+ TB+ patients. Of the 24 HIV+ TB+ patients, 19 (79%) had anti-81 (88)-kDa protein antibodies and 22 (92%) had anti-MPT51 protein antibodies; thus, 92% of the HIV+ TB+ patients had antibodies to either one or both of the antigens. Subclinical TB serum samples from 17 (81%) and 18 (86%) of 21 HIV+ TB+ patients had anti-81 (88)-kDa protein and anti-MPT51 protein antibodies, respectively; together, the two antigens were recognized by 19 (90%) of the 21 HIV+ TB+ patients during subclinical TB (Fig. 1C and D). Anti-81 (88)-kDa protein antibodies were detected in the serum from 1 of 20 HIV+ TB+ individuals. There was no correlation between the presence or absence of antibodies in the serum samples from the HIV+ TB+ patients and their T-cell profiles (Table 1).

The presence of antibodies to these antigens in smear-negative or smear-positive HIV+ TB+ patients and their serum samples obtained during subclinical TB and the absence of antibodies to these antigens in healthy PPDb+ individuals suggest that in vivo \textit{M. tuberculosis} replication is initiated months before the bacterial numbers are high enough to be detectable by AFB smear or culture and that the immune system detects the expression of these antigens. The recognition of the same antigen profile by the subclinical TB serum samples as that recognized by HIV+ TB+ and HIV+ TB+ noncavitary TB patients strengthens our earlier hypothesis that these antigens are expressed by \textit{M. tuberculosis} during intracellular replication in vivo. This is further supported by the fact that 20 of the 21 HIV+ TB+ patients lacked cavitary lesions even at the time of diagnosis of TB and so would have been noncavitary at the time of subclinical TB. The lack of correlation between CD4+ cell counts and the presence or absence of antibodies suggests that either there may be functional differences between the T cells or there may be differences in antigen-specific T cells. The immunodominance of the same antigens in both the HIV+ TB+ and HIV+ TB+ patients provides evidence that clinical isolates from different countries express these proteins in vivo (7, 11, 14–18). Earlier studies with multiple serial serum samples from the same patients had shown that antibodies to the \textit{M. tuberculosis} CFP preparation and to the semipurified native 81 (88)-kDa protein were detectable in serum samples obtained over several months during the time period prior to
Clinical TB (11). Detailed analysis of the reactivity of the multiple serial samples with both of these antigens will allow determination of the kinetics of appearance of these antibodies and their titers during subclinical TB. Moreover, anti-MPT51 protein antibodies were present in a significantly greater proportion of HIV+ TB+ individuals than in HIV− TB− patients (P = 0.0088, Fisher’s exact test). Since a similar difference was not observed with regard to anti-81 (88)-kDa protein antibodies, these results suggest that in vivo expression of MPT51 may be enhanced in immunosuppressed HIV+ individuals. Whether the enhanced expression of this protein is related to the aggressive progression of TB in these individuals remains to be explored.

FIG. 1. Reactivity of fractionated CFPs with pooled serum samples from healthy, PPD skin test-positive individuals (A) and subclinical TB serum samples from HIV+ TB+ individuals (B). Antigens recognized by both serum pools are circled in blue; antigens recognized only during subclinical TB are circled in green. The antigen circled in red is a 38-kDa protein previously shown to be recognized primarily by cavitary TB patients (15). Molecular masses (kilodaltons) are indicated at the left of each panel. Panel C shows the reactivity of serum samples with the purified 81 (88)-kDa (striped bars) and MPT51 (hatched bars) proteins. The solid bars show additive reactivity with both antigens. Panel D shows the reactivity of paired serum specimens obtained from each individual during subclinical TB and at the time of diagnosis of clinical TB. The ΔOD (OD obtained with any serum specimen minus the cutoff, which was calculated as the mean OD of the control group plus 3 standard deviations) for each specimen is plotted.
Seventy-five to 80% of the millions of HIV-infected individuals in sub-Saharan Africa and Southeast Asia are coinfected with *M. tuberculosis*, and TB is the leading cause of mortality and morbidity in these countries (2, 3, 8, 21). Monotherapy with isoniazid is recommended by the Joint United Nations Programme on HIV/AIDS-World Health Organization to reduce the risk of TB in coinfected individuals, but administration of preventive monotherapy requires that active TB be excluded to reduce the risk of development of drug resistance (5, 9, 13). No reliable tests that can identify HIV individuals who eventually developed clinical TB and their immune responses elicited during subclinical TB could provide markers that can identify incipient infection with *M. tuberculosis* before progression to clinical disease occurs. Moreover, the specific expression of these proteins during subclinical TB suggests that they may play an important role(s) in the pathogenesis of progressive infection with *M. tuberculosis*.

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**REFERENCES**


