Delineation of Human Antibody Responses to Culture Filtrate Antigens of Mycobacterium tuberculosis

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This study was undertaken to define the antigens in culture filtrates of actively replicating Mycobacterium tuberculosis that are recognized by antibodies from tuberculosis (TB) patients. Two-dimensional Western blots were probed with sera from healthy controls and TB patients that were preabsorbed with Escherichia coli lysates to deplete cross-reactive antibodies. Antibodies from TB patients recognized 26 of the >100 culture filtrate proteins, and the repertoire changed with disease progression. Only 12 of 26 antigens, including 3 proteins implicated in colonization and invasion by mycobacteria (MPT51, MPT32, and 85C), and 9 (as yet undefined proteins) were reactive with sera from TB patients with early noncavitary or cavitary disease. Eight additional antigens, including 4 undefined proteins, were recognized only by sera from a subset of patients with advanced cavitary disease. Studies suggest that 3 of the antigens recognized by sera from patients with early TB (85C, MPT32, and a 88-kDa protein) have strong serodiagnostic potential.

The presence of cross-reactive antibodies against mycobacterial antigens in sera from healthy persons [1, 2] has hindered definition of the repertoire of antigens that elicits antibodies during active tuberculosis (TB). We have demonstrated that subtractive immunabsorption of sera with Escherichia coli lysates that contain many of the ubiquitous prokaryotic proteins results in significant depletion of cross-reactive antibodies in sera from both healthy persons and TB patients [3], enabling the dissection of humoral responses in TB. By use of absorbed sera, we confirmed earlier reports that only sera from a subset of patients with cavity TB recognize the previously defined 38-kDa (PstS) serodominant antigen, antibodies to which are associated with severe, recurrent disease [4]. We also identified an 88-kDa antigen that elicits antibodies during preclinical TB in patients infected with human immunodeficiency virus (HIV) [5], in noncavitary TB patients, and in cavitary TB patients lacking anti-38-kDa antibodies [3]. These results suggest that anti-88-kDa antibodies appear earlier during disease progression than anti-38-kDa antibodies and show that within the same clinical group, there are different antibody response patterns. Thus, patients with cavitary TB can have both anti-38- and anti-88-kDa antibodies, anti-88-kDa antibodies only, or lack antibodies to both antigens. Noncavitary TB patients have anti-38-kDa, but lack anti-38-kDa antibodies [3], or lack both antibodies.

To define the repertoire of culture filtrate antigens recognized by TB patients and to identify additional antigens that are recognized during early TB, antibody responses in patients with and without anti-38-kDa antibodies were evaluated. Culture-filtrate antigens of M. tuberculosis were fractionated by 2-dimensional (2-D) electrophoresis, and blots were probed with sera from healthy persons and from TB patients. The utility of antigens recognized by sera from patients with early TB for developing serodiagnosis was evaluated.

Materials and Methods

Antigens. The culture filtrate proteins (CFPs) of Mycobacterium tuberculosis H37Rv (liposaccharidomannan [LAM]-free) were prepared as described [3]. This preparation contains >100 proteins [6]. Fractionation of the LAM-free CFP into 15 overlapping fractions and their reactivity with TB and control sera and an extensive panel of anti-M. tuberculosis monoclonal antibodies (World Health Organization) have been described [3]. Reactivity with the 38-kDa PstS (fraction F10) and the seroreactive 88-kDa antigen (fraction F15) was used to classify sera [3]. Purified M. tuberculosis protein Ag85C and MPT32 were prepared as described earlier [7, 8].

Subjects. Serum samples were from 18 healthy controls (12 purified protein derivative skin test–positive, 6 –negative, group 1) and 30 HIV-negative pulmonary TB patients (bled within 1–24 weeks of treatment initiation), who were described previously [3].
Eight TB patients (3 cavitary, 5 noncavitary) lacked antibodies to both the 38-kDa PstS and 88-kDa antigens (group 2). Eleven other TB patients (5 cavitary, 6 noncavitary) had antibodies to the 88-kDa but not to the 38-kDa PstS antigen (group 3). The remaining 11 TB patients had cavitary disease and had antibodies to both the 38- and 88-kDa antigens (group 4).

Immunoblot preparation was done as described [3]. In brief, the antigens (2 µg/mL, 50 µL/well) were coated overnight onto plates (Immulon 4; Dynatech, Alexandria, VA), after which the wells were blocked (7.5% fetal bovine serum; Hyclone, Logan, UT, and 2.5% bovine serum albumin [BSA]). Diluted sera (50 µL/well: 1:150 for MPT32 and 1:50 for 85C) was added for 90 min at 37°C, then the plates were washed, and 50 µL/well alkaline phosphatase-conjugated anti-human IgG (1:2000 dilution; Zymed, San Francisco) was added for 60 min. The plates were washed with Tris-buffered saline (50 mM Tris, 150 mM NaCl) and developed with an amplification system (Life Technologies Gibco BRL, Gaithersburg, MD). The mean optical density (490 nm) ± 3 SD obtained with sera from the healthy controls with each antigen was used as the cutoff.

2-D SDS-PAGE and Western blotting. 2-D PAGE and immunoblots were prepared as described [6]. In brief, 70 µg of LAM-free CFP resuspended in 30 µL of isoelectric focusing (IEF) sample buffer (9 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol, and 5% pharmalytes, pH 3–10) was incubated for 3 h at 20°C, and 25 µL was applied to a 6% polyacrylamide IEF tube gel containing 5% pharmalytes, pH 3–10 and 4–6.5 at a 1:4 ratio, and focused for 3 h at 1 kV. The tube gels were electrophoresed (2-D) on 15% SDS-PAGE at 20 mA/gel for 0.3 h followed by 30 mA/gel for 1.8 h. Blots prepared from the gels were blocked with 3% BSA in PBS, washed with PBS–TWEEN 2%, and probed with the 4 serum pools (1:200 dilution) prepared from 6 persons from each subject group described above. The alkaline phosphatase-conjugated anti-human IgG was used at 1:2000 with BCIP–nitroblue tetrazolium substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Results

A 2-D map of all M. tuberculosis CFPs was recently generated on the basis of reactivity with anti-M. tuberculosis murine monoclonal antibodies (MAbs) and peptide sequence analyses of several antigens [6]. To map the proteins recognized by TB patients, similar 2-D immunobLOTS of LAM-free CFPs were probed with the 4 serum pools, and the reactivity obtained was compared with the 2-D map.

The antigens reactive with the 4 serum pools are shown in figure 1. The known antigens are identified; for the undefined antigens (A—M), the reference numbers previously assigned [6] are provided in the legend. The serum pools from groups 1–4 reacted with 6, 4, 18, and 26 antigens, respectively (figure 1).

All 4 serum pools reacted with 4 culture-filterate antigens, including the ~31-kDa 85A, ~29-kDa 85B (both reactive with MAb IT-49), ~55-kDa glutamine synthetase, and an undefined ~58-kDa antigen (B). Two antigens, MPT64 (IT-67) and a protein marked “A,” were reactive with serum pools from groups 1, 3, and 4, but not group 2.

In addition to these 6 antigens, the group 3 serum pool reacted with 12 other antigens (figure 1C). Eleven of these 12 antigens (except “C”) were also reactive with group 4 sera. Of the antigens that were reactive with both group 3 and 4 serum pools, 4 were identified: ~26-kDa MPT51 (IT-52), ~31-kDa 85C (IT-49), ~40–42-kDa MPT32, and an 85–88-kDa protein cluster reactive with MAb IT-57 (~88-kDa seroreactive antigen defined earlier [3]). One of the 4 isoforms of the 38-kDa PstS protein (IT-23) also showed weak reactivity with the group 3 pool (figure 1C), even though none of the individual sera in this pool reacted with this antigen by ELISA (with F10), probably due to low titers of anti-38-kDa antibodies in individual sera. The remaining antigens that reacted with both group 3 and 4 pooled sera are unidentiﬁed. In contrast to the group 3 pool, the group 4 serum pool showed strong reactivity with all 4 isoforms of the 38-kDa protein (figure 1D) and recognized several additional antigens, including the GroES (IT-3), DnaK (IT-41), and 4 unidentified proteins.

These results suggest that antigens such as 85C, MPT32, MPT51, and the 88-kDa protein that are recognized by both group 3 and 4 pools should be potential candidates for development of serodiagnosis, since both cavitary and noncavitary TB patients have antibodies to them. Antigens recognized only by group 4 pool (e.g., 38-kDa PstS) would be recognized by sera from fewer patients with TB.

To determine the utility of antigens identified by both group 3 and 4 serum pools for serodiagnosis, reactivity with purified 85C [7] and MPT32 [8] was assessed. Reactivities with the 88-kDa (F15) and 38-kDa PstS (F10) antigens with the same cohort are also shown (figure 2). Sixteen of 19 cavitary and 5 of 11 noncavitary patients had anti–88-kDa antibodies, whereas only 11 cavitary patients had anti–38-kDa antibodies. Seventeen cavitary and 6 noncavitary patients had antibodies to 85C, and 16 cavitary and 1 noncavitary patients had antibodies to MPT32 (figure 2).

Discussion

These results show that of the >100 proteins in the culture filtrates of M. tuberculosis replicating in bacteriologic media in vitro [6], only 26 proteins are recognized by antibodies from TB patients. This suggests that not all proteins secreted in vitro in culture filtrates may be expressed in significant amounts in vivo during active disease. Only the group 4 serum pool reacted with all 26 antigens (figure 2D). Since anti–38-kDa antibodies have been associated with advanced, recurrent, and chronic TB, the results suggest that the other antigens recognized exclusively

Discussion
Figure 1. Two-dimensional fractionation and immunoblot analysis of *M. tuberculosis* LAM-free culture-filtrate proteins (CFPs). Blots were probed with pooled sera from persons in: A, group 1, purified protein derivative-positive healthy controls; B, group 2, TB patients lacking both anti-38- (PstS) and anti-88-kDa antibodies; C, group 3, TB patients with anti-88- but no anti-38-kDa antibodies; and D, group 4, TB patients with both anti-88- and anti-38-kDa antibodies. Identities of known proteins and/or reactive murine monoclonal antibodies are in parentheses. Reference numbers of proteins (A–M) based on [6] are as follows: A, not assigned; B, 86, 96, 105; C, 77; D, 59, 69; E, 103; F, 68, 80; G, 24; H, not assigned; I, 111; J, 57; K, 62; L, 32; M, 78.

by this serum pool may be expressed in significant amounts in vivo only during advanced TB [4]. The reactivity of group 3 sera with only 18 of 26 proteins suggests that fewer antigens may be expressed in vivo during relatively early disease.

Protein profiles expressed in vivo by *M. tuberculosis* may differ from those expressed during in vitro extracellular or intracellular growth and may also vary with the local milieu in which the bacteria replicate in vivo. Significant extracellular replication of *M. tuberculosis* can be seen in cavitary lesions [10], but the extent of cavitation and extracellular replication of in vivo bacteria does not always correlate [11]. The group 4 serum pool, which included sera from only cavitary patients with anti-38-kDa antibodies, recognized the largest number of antigens in culture filtrates from extracellularly replicating bacteria. This may reflect more liquefaction and in vivo extracellular bacterial replication in these patients. Of interest, the 3 known antigens reactive exclusively with the group 4 pool (GroES, DnaK, PstS) are all expressed by *M. tuberculosis* in vitro during adverse environmental conditions.

In contrast, all 3 known proteins recognized by the group 3 pool are implicated as virulence factors for *M. tuberculosis*: MPT32 is homologous to a fibronectin-binding protein of *Mycobacterium leprae* (43 L) believed to be involved in invasion of epithelial and Schwann cells [12], and 85C and MPT51 belong to the fibronectin-binding protein family of mycobacteria [13]; the former has mycolyltransferase activity [7]. These proteins may be secreted by the bacteria relatively earlier during the course of disease progression, before the development of
Figure 2. Reactivity of sera from TB-negative, human immunodeficiency virus-negative, purified protein derivative-positive healthy controls (■); cavity TB patients (●), and noncavitary TB patients (▲) with (A) sized-fraction 15 (F15) containing 88-kDa antigen [3], (B) sized-fraction 10 (F10) containing 38-kDa antigen [3], (C) purified antigen 85C [7], and (D) purified MPT32 [8]. Dashed lines represent mean ± 3 SD of control group.

extensive cavitation or in vivo extracellular replication. Of interest, serum antibodies from diseased persons confirmed in vivo expression of virulence factors of several other bacterial pathogens (e.g., IpaB, -C, and -D of *Shigella flexneri*, internalin and listeriolysin of *Listeria monocytogenes*, the secreted proteinases and the iron-regulated proteins of *Trichomonas vaginalis*, neuraminidase of *Pasteurella haemolytica*, and Vero cytotoxin-1 of *E. coli*; reviewed in [14]).

The control sera showed reactivity with 6 antigens, and 2 of these, 85A and 85B, are members of the fibronectin-binding family of proteins [15]. Antigen 85 homologues are present in nonpathogenic mycobacteria and in corynebacteria [13], and absorption with *E. coli* lysates may fail to totally eliminate the cross-reactive antibodies elicited by a variety of other bacteria. However, the stronger reactivity of TB sera with these antigens suggests that the mycobacterial proteins possess specific serodominant epitopes.

Antibodies are markers of antigens expressed in vivo, and such antigens are likely to be the best reagents for diagnosis and immunization. The reactivity of sera with antigen 85C, MPT32, and the 88-kDa antigen confirms the serodiagnostic potential of proteins that are reactive with both group 3 and 4 pools (figure 2). Although larger cohorts of patients and controls from different geographic locations need to be tested, these data show the utility of focusing on in vivo–expressed antigens. Identification of the other immunogenic proteins, and cellular studies with these in vivo–expressed antigens are required to gain insight into the mechanisms of pathogenesis and disease progression.

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References


