Disease State Differentiation and Identification of Tuberculosis Biomarkers via Native Antigen Array Profiling*S

Mark J. Sartain‡, Richard A. Slayden‡, Krishna K. Singh§, Suman Laal¶, and John T. Belisle††

A critical element of tuberculosis control is early and sensitive diagnosis of infection and disease. Our laboratories recently showed that different stages of disease were distinguishable via two-dimensional Western blot analyses of Mycobacterium tuberculosis culture filtrate proteins. However, this methodology is not suitable for high throughput testing. Advances in protein microarray technology provide a realistic mechanism to screen a large number of serum samples against thousands of proteins to identify biomarkers of disease states. Techniques were established for separation of native M. tuberculosis cytosol and culture filtrate proteins, resulting in 960 unique protein fractions that were used to generate protein microarrays. Evaluation of serological reactivity from 42 patients in three tuberculosis disease states and healthy purified protein derivative-positive individuals demonstrated that human immunodeficiency virus (HIV)-negative cavitary and noncavitary tuberculosis (TB) patients' sera recognized 126 and 59 fractions, respectively. Sera from HIV patients coinfected with TB recognized 20 fractions of which five overlapped with those recognized by non-HIV TB patients' sera and 15 were unique to the HIV+ TB+ disease state. Identification of antigens within the reactive fractions yielded 11 products recognized by both cavitary and noncavitary TB patients' sera and four proteins (HspX, MPT64, PstS1, and TrxC) specific to cavitary TB patients. Moreover four novel B cell antigens (BfrB, LppZ, SodC, and TrxC) of human tuberculosis were identified. Molecular & Cellular Proteomics 5: 2102–2113, 2006.

Recent World Health Organization data show that the global incidence of tuberculosis (TB) year and that there are an estimated 8.8 million new cases each year (1). One concerning fact is that less than half of the 8.8 million estimated cases are diagnosed as smear-positive. This underscores the need for a rapid, sensitive diagnostic test to aid TB control efforts. The development of such a test has proven to be one of the greatest challenges in TB research.

In recent years there has been renewed interest in developing antibody-based diagnostics that utilize multiple antigens to achieve high levels of sensitivity and specificity (2). The success of a serodiagnostic test for TB hinges on its ability to detect multiple disease states, including pauci- and multibacillary forms, pediatric cases, and patients coinfected with human immunodeficiency virus (HIV). Previous work from our laboratories identified several antigens that provide high sensitivity and specificity when used in an ELISA format (3–9). Furthermore this work highlighted differential antigen reactivity based on the disease state (3, 5, 6, 8–10). However, a complete analysis of patients' serological reactivity to a large proportion of the Mycobacterium tuberculosis (Mtb) proteome is hindered by the inability to evaluate the reactivity of the nearly 4,000 predicted proteins of Mtb in a high throughput fashion.

Over the past several years, protein microarrays have shown considerable potential for detecting antigen-antibody interactions on a proteomic scale (11–13). As a proof of principle, Bacarese-Hamilton et al. (14) immobilized recombinant antigens of various pathogens to glass slides, and human antibodies specific for each antigen were detected in subpicogram amounts. This microarray assay also performed at the same level of efficiency as conventional ELISA-based methods in differentiating between positive and negative sera. Microarray technology has now been extended to characterize antibody responses generated upon vaccination with Yersinia pestis (15) and vaccinia virus (16). Protein microarrays accommodate thousands of individual antigens or antigen
pools on a single slide, and automation allows for hundreds of slides to be generated at once. Moreover this methodology is facile and allows reproducible screening of sera from a large number of individual patients.

In the absence of a complete *Mtb* open reading frame library, methods to produce a first generation *Mtb* protein microarray based on native proteins were required. A multidimensional separation strategy was devised to efficiently resolve native proteins found in the cytosol and culture filtrate of *Mtb*. This resulted in 960 relatively simple protein fractions from two highly complex protein pools. These fractions were spotted to nitrocellulose slides and probed with sera from purified protein derivative-positive (PPD+) healthy controls, cavitary TB, noncavitary TB, and HIV- and *Mtb*-coinfected patients. The resulting analyses corroborated our earlier two-dimensional (2-D) immunoblot based experiments, confirming that a subset of antigens is recognized early in TB disease progression. Furthermore four proteins specific for cavitary TB patients were identified, and four novel antigens previously undetected by other methods were defined as serodiagnostic targets.

**EXPERIMENTAL PROCEDURES**

**Preparation of *Mtb* Subcellular Fractions**

*Mtb* strain H37Rv was expanded from a 1-ml frozen stock (−10⁸ colony-forming units/ml) to 24 liters of late log culture in glycerol-alanine salts medium (17). The culture supernatant was separated from the cells and processed to generate the culture filtrate proteins (CFPs) of *Mtb* as described previously (18). The *Mtb* H37Rv cells (88.9 g, wet weight) were washed three times with PBS (pH 7.4), frozen at −70 °C, and inactivated with 24 kilograms of γ-irradiation. Lysis of these cells was achieved by suspending them in 44 ml of TSE buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA) containing 0.06% DNase, 0.06% RNase, 0.07% peptatin, 0.05% leupeptin, and 20 μM PMSF and passing them through a French press five times at 1,500 p.s.i. The resulting lysate was diluted with 1 volume of TSE buffer and centrifuged at 2,000 × *g* for 5 min to remove unbroken cells. The cytosol was obtained as the final supernatant of sequential centrifugations at 27,000 × *g* and 100,000 × *g* (19) and was dialyzed against 10 mM ammonium bicarbonate using a 3,500-Da molecular mass cutoff membrane. The protein concentrations of the cytosol and CFP were determined with the bicinchoninic acid (BCA) protein assay (20).

**Multidimensional Protein Fractionation**

Initial fractionation of the CFP (124 ml at 3.6 mg/ml) and cytosolic proteins (200 ml at 2.5 mg/ml) was achieved with sequential rounds of ammonium sulfate precipitation. Specifically the CFP and cytosolic proteins were precipitated with 42 and 67% and 29 and 44% saturated ammonium sulfate, respectively. Precipitated proteins were collected by centrifugation at 10,000 × *g* for 4 °C for 1 h. All protein pellets were suspended in 20 ml Tris-HCl (pH 8.0). These suspensions and the final supernatants of the sequential precipitations were dialyzed against 20 mM Tris-HCl (pH 8.0) using a 3,500-Da molecular mass cutoff membrane, and the dialyzed protein solutions were concentrated where needed. To ensure the removal of contaminating nucleic acids, MgCl₂ (5 mM final concentration) and DNase and RNase (1.25% final concentration) were added to each fraction followed by incubation at 37 °C for 30 min.

The fractions obtained by ammonium sulfate precipitation were adjusted to 10% acetonitrile and applied to an HPLC column (1 × 10 cm) packed with Source 15Q strong anion exchange (AIEX) resin (Amersham Biosciences). Proteins were eluted with a step gradient of increasing concentrations of NaCl at a flow rate of 3.3 ml/min using a Waters 600E HPLC system (Waters Corp., Milford, MA). The eluted protein fractions were concentrated 100-fold and exchanged into 20 mM ammonium bicarbonate by ultrafiltration. Protein concentrations were determined by the BCA assay, and fractions containing less than 1 mg of protein were pooled. All other fractions were kept separate. The concentrated AIEX fractions were adjusted to 10% acetonitrile and applied to an HPLC Source™ 15RPC ST 4.6/100 column (Amersham Biosciences), and the proteins were eluted with an increasing linear gradient (10–70%) of acetonitrile. All fractions were dried in a SpeedVac and suspended in 67 μl of 10 mM ammonium bicarbonate, and protein concentrations were determined by the BCA assay.

**Human Sera and Antibodies**

Sera from the following groups of individuals were obtained with informed consent.

(i) Twelve PPD-positive Healthy Individuals—Seven of these individuals were recent immigrants from countries where *Mtb* is endemic, many of whom had been vaccinated with *Mycobacterium bovis* strain Bacille Calmette-Guerin; the remaining five individuals were from the United States or western Europe and were not vaccinated with the Bacille Calmette-Guerin strain.

(ii) Nine Noncavitary TB Patients with No Recognizable Cavitary Lesions on Chest X-rays—These were acid-fast bacilli sputum smear-negative (six of nine) or -positive (three of nine), culture-positive patients attending the infectious disease clinic at the Manhattan Veterans Affairs Medical Center. None of these patients were HIV-infected. These individuals were bled either prior to or within 2 weeks of the initiation of therapy for TB.

(iii) Eleven Cavitary TB Patients with Moderate-to-advanced Cavitary Lesions as Determined by Chest X-rays—These were sputum smear-, acid-fast bacilli-positive patients obtained from the Lala Ram Sarup Institute in New Delhi, India who were all bled prior to initiation of therapy for TB. None of these patients were HIV-infected.

(iv) Ten HIV-positive TB Patients—These were sputum smear-positive (seven of 10) or -negative (three of 10), culture-confirmed patients from the Manhattan Veterans Affairs Medical Center. None of the patients had radiological evidence of cavitary lesions. All 10 patients were known to possess antibodies to the CFP of *Mtb* when tested by ELISA in earlier studies (3, 6). These patients were bled either prior to or within 2 weeks of the initiation of therapy for TB.

(v) Six HIV-positive TB-negative Patients—These were asymptomatic, HIV-infected individuals from the Manhattan Veterans Affairs Medical Center. All sera were preadsorbed with *Escherichia coli* lysates to remove cross-reactive antibodies to ubiquitous pokyaryotic proteins as described earlier (4).

Monoclonal antibodies (mAbs) and polyclonal sera against specific *Mtb* proteins were obtained from the Colorado State University Tuberculosis Research Materials and Vaccine Testing Contract (NIAID, National Institutes of Health Contract NO1 AI-75320). The following antibodies and dilutions were used for both microarray analyses and immunoblots: IT-12 α-19 kDa (1:20), IT-20 α-HspX (1:100), IT-23 α-PstS1 (1:20), IT-47 α-PstS1 (1:20), IT-52 α-MPT51 (1:5), CS-35 α-LAM (1:20), CS-49 α-HspX (1:100), CS-93 α-45 kDa (1:20), and α-45 kDa polyclonal sera (1:1,000).

**Protein Microarray Printing and Probing**

An aliquot (5 μg of protein) of each multidimensional chromatography fraction was transferred to 384-well microtiter plates, dried, and
solubilized in 25 µl of FAST® protein array print buffer (Schleicher & Schuell). The plates were centrifuged briefly (2,000 × g) to pellet any precipitate, and ~1 nl of the supernatants (0.2 mg/ml) was printed to nitrocellulose-coated FAST glass slides (Schleicher & Schuell) using Stealth SMP3® spotting pins (TeleChem International, Sunnyvale, CA) and a VersArray® Chipwriter Pro microarray contact printer (Bio-Rad). Cytosolic proteins, CFP, the native 38-kDa PstS1 protein (Rv0934), and the six ammonium sulfate precipitation fractions were also printed in a dilution series of 1.6, 0.8, 0.4, 0.2, 0.1, 0.5, 0.25, and 0.125 mg/ml. As negative controls, E. coli whole-cell lysate (WCL) was printed in the same dilution series, and FAST print buffer was printed alone. All samples were printed in triplicate, resulting in 3,768 total spots per slide. The slides were allowed to dry 1 h at room temperature (RT) and stored at 4 °C until use. Printed microarray slides were washed 10 min in commercial FAST protein array wash buffer at RT and stored at 4 °C until use. Printed microarray slides were washed 10 min in commercial FAST protein array wash buffer (Schleicher & Schuell), and probed with individual serum (750 µl) diluted 1:100 in PBS (pH 7.4), 1% BSA for 1 h at RT. Slides were washed twice for 10 min, allowed to dry, and scanned using a VersArray Chipreader (Bio-Rad). Probing of the microarray slides with mAbs or polyclonal sera was performed in the same manner, except Cy3-conjugated anti-human IgG (Sigma, St. Louis, MO) diluted 1:500 in FAST protein array wash buffer. Slides were again washed twice for 10 min, allowed to dry, and scanned using a VersArray Chipreader (Bio-Rad). Microarray analyses of individual patient’s serum were repeated in triplicate, and one slide was used for each mAb or rabbit polyclonal sera.

Microarray Data Analyses

Microarray spot intensity values were quantified with The Institute for Genomic Research Spotfinder software (21). Signal-to-noise ratios (SNRs) were calculated for each spot by dividing the raw intensity (sum of all pixels per spot) by the background intensity (local background median multiplied by spot area). Analysis of SNR reduced the local background variation or bias observed between individual slides. The mean SNR for each protein or protein fraction printed in triplicate was determined, resulting in an averaged SNR (AvSNR). To allow for direct patient-to-patient or slide-to-slide comparisons, all AvSNRs for a slide were normalized against the median AvSNR of all multidimensional chromatography fractions of the slide. The normalized AvSNR (NavSNR) was determined by dividing the mean AvSNR by the median AvSNR because the median AvSNR was less affected by variations in reactivity between sera. For the microarray slides probed with mAbs or rabbit polyclonal sera, the AvSNR for each fraction was calculated.

SDS-PAGE and Western Blot Analyses

SDS-PAGE of multidimensional chromatography fractions was performed with 10–20% polyacrylamide gradient Tricine gels (Invitrogen) or 15% Tris-glycine gels (10 × 7.5 cm) (22). Protein staining was achieved with silver nitrate (23) or Coomassie Brilliant Blue R-250. For Western blot analyses, aliquots (3 µg) of selected fractions were resolved on 10–20% polyacrylamide gradient Tricine gels and electroblotted to nitrocellulose membranes (17). The membranes were blocked with 3% nonfat milk in PBS (pH 7.2) for 2 h, washed with PBS containing 2% Tween 20, and exposed overnight to preabsorbed pooled sera from patients and control subjects diluted 1:200. The blots were washed with PBS containing 2% Tween 20, probed with alkaline phosphatase-conjugated anti-human IgG (1:2,000, Sigma) for 1.5 h, and washed extensively. Antigen-antibody complexes were visualized by color development with 5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Mass Spectrometry

Coomassie-stained protein bands corresponding to those that reacted to patients’ sera on Western blots were excised and subjected to in-gel digestion with modified trypsin (Roche Applied Science), and the resulting peptides were extracted with 60% acetonitrile, 0.1% TFA (24). Extracted peptides were applied to a capillary (0.2 × 50-mm) C18 reversed phase (RP) column (Microchrom BioResources, Auburn, CA) and eluted with an increasing linear gradient (5–70%) of acetonitrile in 0.1% acetic acid using an Eldex MicroPro capillary HPLC system (Napa, CA) with a flow rate of 5 µl/min. The RP eluant was introduced directly into a ThermoFinnigan LCQ electrospray mass spectrometer (San Jose, CA) using Xcalibur software version 1.3, and the peptides were analyzed by MS/MS. The electrospray needle was set at 4 Kvol with a N2 sheath gas flow of 40 and a capillary temperature of 200 °C. MS/MS was automatically performed on the most dominant ion of the previous scan, and the normalized collision energy was set at 40%. BioWorks 3.1 turboSEQUEST software (ThermoFinnigan) was used to match the MS/MS data of peptide to protein sequences extracted from the Mtb genome database (NC_000962) that contained 3,989 proteins. The software was set to evaluate peptide obtained by trypsin or chymotrypsin and Glu-C digestion and to consider the oxidation of methionine (+16.0 amu) and the acrylamide modification of cysteine (+71.0 amu). The Scaffold software (Proteome Software, Portland, OR) that verifies peptide identifications made by SEQUEST and probabilistically validates the peptide and protein identifications was applied to all MS/MS sequencing results.

RESULTS

Multidimensional Protein Fractionation Resulted in the Reduced Complexity of Mycobacterial Protein Pools—Recent advances in proteomics have resulted in the ability to separate and identify individual proteins or peptides from complex biological samples. Specifically multidimensional chromatography of peptides, derived from tryptic digests of crude biological samples, followed by MS/MS analysis has resulted in the experimental validation of substantial portions of theoretical proteomes (25). Recently this approach was applied to Mtb and resulted in more than a 3-fold increase in the number of proteins previously identified by 2-D gel electrophoresis-based methods (26). With goals similar to the MudPIT strategy, this study used a multidimensional fractionation scheme (Supplemental Fig. S1) to efficiently separate complex pools of intact mycobacterial proteins into relatively simple and enriched fractions that could be evaluated for serological reactivity in a high throughput fashion. Aliquots of cytosolic proteins (500 mg) and CFPs (446 mg) from Mtb H37Rv were subjected to sequential ammonium sulfate precipitations, resulting in six fractions with nearly equal protein amounts (Fig. 1A). Subsequent AIEX chromatography expanded the fraction number to 78 with protein yields varying from 160 µg to 13 mg per fraction (Fig. 1B). Those fractions containing 500 µg of protein were excluded from further separation (eight fractions), and fractions containing between 500 µg and 1 mg were pooled with neighboring fractions (10
fractions). This resulted in a total of 64 fractions that were applied to RP chromatography under mildly basic pH conditions. This multidimensional chromatography separation yielded a total of 960 fractions, and SDS-PAGE analysis revealed varied complexity among the fractions with one to >20 proteins observed per fraction (Fig. 1C).

Validation of the Protein Microarray Format—Each multidimensional chromatography fraction as well as intermediate fractions and recombinant proteins was printed to nitrocellulose microarray slides. Sera from PPD+ healthy (n = 12), noncavitary TB (n = 9), cavitary TB (n = 11), and HIV+TB+ (n = 10) individuals were probed against the microarrays in triplicate, and for each slide the NAvSNR (see “Experimental Procedures”) was calculated for each protein or protein fraction. The integrity of the protein microarray and validation of this platform was determined by assessing the reactivity of TB patients’ sera with selected protein fractions and purified proteins spotted on the microarray slides as controls and comparing these data with published results obtained by plate ELISA (5, 6). To demonstrate specificity, the reactivity of TB patients’ sera to unfractionated CFP was compared with that of E. coli WCL and FAST print buffer. The CFP is known to contain numerous B cell antigens (2), and as expected, cavitary TB patients’ sera displayed significantly greater reactivity to CFP than to either buffer alone (p value = 0.001) or E. coli WCL (p value = 0.002) (Fig. 2A). Although patient-to-patient variability in reactivity to unfractionated CFP was observed, each individual cavitary TB patient’s serum recognized CFP more strongly than they did buffer or E. coli WCL.

A second validation control was performed with purified native 38-kDa protein PstS1/Rv0934, a previously characterized B cell antigen (2). Reactivity to PstS1 was compared among the four patient groups used in this study (Fig. 2B). Sera from TB patients showed greater reactivity than that from PPD+ healthy individuals. Furthermore when evaluated using the mean NAVSNR of PPD+ healthy controls plus 3 times the standard deviation (S.D.) as a cutoff, the number of patients’ sera demonstrating a positive response to PstS1 was greatest for cavitary TB (72%) followed by noncavitary TB (33%) and HIV+TB+ (30%) patients. These results concurred with data previously obtained via traditional ELISA platforms (5, 6). The PstS1 was also printed at multiple concentrations, and a dose-dependent antibody response was observed (data not shown).

TB Disease States React to a Defined Group of Antigens—To evaluate patterns of serological reactivity the NAVSNR values of each fraction were averaged for all patients within a disease state and expressed as a ratio over the averaged PPD+ healthy control NAVSNR of the corresponding fraction (Fig. 3A). Fractions originating from the CFP (fractions 1–525) were more antigenic overall than those derived from the cytosol (fractions 526–960). Additionally differences in reactivity based on disease state were observed. This was most pronounced when comparing HIV+TB+ patients to cavitary or noncavitary TB patients. The HIV+TB+ sera generally displayed poor reactivity except for a distinct cluster of CFP fractions that were not well recognized by sera from other disease states (Fig. 3A).

To simplify patterns of antigen reactivity and to further assess similarities and differences in antigen recognition between disease states, a cutoff value of the mean NAVSNR of PPD+ healthy controls plus 3 × S.D. was established for each fraction. Fractions displaying NAVSNR values greater than the cutoff value in 40% or more of the individuals in a disease state were selected and organized in a Venn diagram (Fig. 3B). Using these criteria, 145 of the 960 fractions (15%) were identified as having significant serological reactivity. As shown in Fig. 3B, cavitary TB patients’ sera recognized 126
fractions, whereas noncavitary TB patients' sera showed significant reactivity to 59 fractions of which 55 were also recognized by cavitary TB patients. The remaining four fractions were unique to noncavitary TB patients. The pattern of fractions recognized by HIV/TB patients was less complex than that of either cavitary or noncavitary patients, and the overlap with these other two patient groups was minimal (Fig. 3B). HIV/TB patients' sera recognized 20 fractions of which five overlapped with those recognized by cavitary TB patients' sera, and of this latter group two also reacted to noncavitary TB patients' sera. Interestingly the 15 fractions that demonstrated significant reactivity to only HIV/TB patients' sera possessed very similar separation characteristics. 1) They originated from the CFP and did not precipitate with 67% ammonium sulfate, and 2) they bound strongly to the AEX column but weakly to the RP column. In Fig. 3A these 15 fractions correspond to the cluster of CFP fractions that showed enhanced reactivity with HIV/TB patients' sera. To confirm that the antigen(s) in these fractions was specific to TB rather than HIV infection, the reactivity of three of the 15 fractions was assessed with HIV/TB− patients' sera, and no antibody responses significantly greater than that of PPD+ healthy controls were observed (data not shown).

Antigen Identification of Reactive Fractions Confirmed the Reactivity to Known B Cell Antigens—To identify individual antigens with the greatest utility in a serodiagnostic assay additional stringency was applied to the array data, further reducing the number of fractions demonstrating significant reactivity from 145 to 105. This was achieved by restricting analyses of the cavitary TB-specific fractions to those that yielded significant serological reactivity (>3× S.D. above PPD+ mean) with 55% (six of 11) or greater of cavitary TB patients.

It was also recognized that lipoarabinomannan (LAM), a well characterized B cell antigen (27), would be present in some of the multidimensional fractions and was expected to be serologically dominant. Thus, microarray slides were probed with the CS-35 mAb specific for LAM (Fig. 4). Of the 105 targeted fractions 24 were found to contain LAM. The serological dominance of LAM was confirmed with conventional one-dimen-

Fig. 2. Validation of protein microarray integrity. A, reactivity of cavitary TB patients' sera against buffer (●), E. coli WCL (▲), and Mtb CFP (▼). B, reactivity of PPD+ healthy controls (●), HIV+TB+ (▼), noncavitary TB (□), and cavitary TB (▲) patients' sera against spotted native, purified 38-kDa PstS1. The patient-averaged NAvSNR +3× S.D. was obtained by use of sera from healthy PPD+ control subjects, shown as the horizontal dashed line, and was used as the cutoff to determine positive reactivity. Noncav. noncavitary.

Fig. 3. Global analysis of HIV+TB+, noncavitary TB, and cavitary TB patient reactivity against all 960 protein fractions. A, the NAvSNR of each fraction was averaged for all patients in each disease state and expressed as a ratio over the averaged healthy PPD+ NAvSNR of the corresponding fraction. Culture filtrate fractions, 1–525; cytosolic fractions, 526–960. B, a Venn diagram displaying the number of fractions with significant reactivity to sera from each disease state and the relatedness of these serological responses. The fractions included in the Venn diagram were recognized by ≥40% of patients. The asterisk indicates a unique set of fractions recognized by HIV+TB+ patients. CF, culture filtrate; CYT, cytosol.
probed with pooled cavitary TB patients’ sera. LAM-specific mAb CS-35. The arrays probed with serum from a single cavitary TB patient and the mannan as the serodominant antigen.

Western blot analyses with patients’ sera compared alongside methods (Supplemental Fig. S1). The first of these utilized proteins within the remaining fractions were obtained by two eluding from further antigen analyses.

Molecular identities of the serologically active native proteins within the remaining fractions were obtained by two methods (Supplemental Fig. S1). The first of these utilized Western blot analyses with patients’ sera compared alongside the reactivity to mAbs or polyclonal sera specific for five 

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Ten proteins previously found to be human B cell antigens (2) accounted for all or part of the serological activity of 26 fractions (Table II). mAbs were used as the sole evidence to identify the presence of the 38-kDa PstS1 antigen, the 19-kDa lipoprotein antigen, and the 14-kDa HspX antigen in four reactive fractions. Specifically the probing of a microarray slide with mAbs IT-23 and IT-47 identified PstS1 in fraction C-II-11. Western blot analyses with IT-47, cavitary TB sera, and PPD+ healthy control sera also revealed a single protein that reacted with IT-47 and cavitary-TB patients’ sera but not sera of PPD+ healthy control individuals (Fig. 5A). In this same manner, the 19-kDa protein was found to be a reactive product of fractions B-I-7 and C-I-14, and HspX contributed to the reactivity of fraction B-V-9. It was also noted that fractions B-I-7 and C-I-14 each contained a second reactive product of 12 and 45 kDa, respectively, and fraction B-V-9 contained two additional reactive proteins of 7 and 10 kDa. The identity of these unknown proteins was not determined due to insufficient protein quantities.

The remaining seven previously described protein antigens (45-kDa Apa, 30-kDa Ag85A and -B, GlcB, Rv3881c, SecE2, and MPT64) were identified by MS/MS analyses, and some of these were confirmed by reactivity to mAbs (Table II and Supplemental Table S1). For some fractions, after Western blot analyses with patients’ sera a sufficient amount of material was not available for protein identification. Thus, protein identity was obtained by MS/MS analysis of an adjacent fraction possessing a reactive band at the same molecular weight. This was done to identify the 45-kDa Apa in one fraction, Ag85B in four fractions, Ag85A in two fractions, GlcB in four fractions, SecE2 in three fractions, and Rv3881c in one fraction (Table II).

Discovery of Novel B Cell Antigens with Potential Serodiagnostic Roles—The MS/MS-based approach to antigen identification led to the elucidation of four new Mtb B cell antigens (Table III). The first of these, SocC (Rv0432), a 27-kDa Cu,Zn-superoxide dismutase (28), was the sole reactive constituent of four fractions originating from the CFP pool (B-III-3, B-III-4, B-IV-3, and B-IV-4) and was significantly recognized by both noncavitary (five of nine) and cavitary TB (eight of 11) sera (Fig. 5B). MS/MS analysis of the corresponding protein in fraction B-III-4 resulted in 42% amino acid coverage of the predicted protein sequence encoded by ORF rv0432 (Fig. 5C).

LppZ (Rv3006) was identified as the only reactive product of five fractions (A-III-5, A-IV-4, A-IV-5, A-IV-6, and A-V-5) originating from the CFP pool and was strongly recognized by noncavitary (four of nine) and cavitary TB (six of 11) sera. These fractions were generated under similar separation conditions, and SDS-PAGE showed all possessed a dominant 45-kDa product. MS/MS analysis of the corresponding protein in fraction A-IV-5 resulted in 37% amino acid coverage of the predicted protein sequence encoded by ORF rv3006.

Bfrb (Rv3841), a putative iron storage protein (29) and a previously described T cell antigen (30), was identified as the reactive protein in two fractions (A-VII-1 and A-VIII-1) with significant reactivity to four of nine noncavitary and six of 11

Fig. 4. Identification of reactive fractions containing lipoarabinomannan as the serodominant antigen. Shown are representative arrays probed with serum from a single cavitary TB patient and the LAM-specific mAb CS-35. The inset is a Western blot of fraction C-I-1 probed with pooled cavitary TB patients’ sera. CAV, cavitary.
### Table I

**Patient reactivity against LAM-containing fractions**

| Fractionation conditions (X,Y,Z)\(^a\) | LAM reactivity ratio\(^b\) | PPD+ | HIV+TB+ | Noncavitary TB | Cavitory TB |
|-----------------------------------------|---------------------------|------|---------|---------------|------------|
| A-III-4                                 | 1.30                      | 1.83 | 2.67    | 3.40          |
| A-IV-1                                  | 3.08                      | 3.49 | 5.71    | 6.31          |
| A-IV-3                                  | 1.28                      | 1.81 | 2.87    | 3.57          |
| A-V-1                                   | 1.67                      | 2.45 | 3.71    | 4.92          |
| A-X-I                                   | 1.41                      | 2.48 | 3.33    | 3.94          |
| A-XI-II-1                               | 1.06                      | 1.49 | 1.74    | 1.99          |
| B-III-I                                 | 1.30                      | 1.49 | 2.71    | 3.12          |
| B-III-7                                 | 0.99                      | 1.38 | 1.99    | 2.06          |
| B-IV-1                                  | 2.30                      | 2.61 | 4.33    | 5.72          |
| B-I-2                                   | 1.23                      | 1.53 | 2.43    | 3.23          |
| C-III-1                                 | 1.18                      | 1.41 | 2.14    | 2.19          |
| C-IV-1                                  | 1.00                      | 1.12 | 1.59    | 1.36          |
| C-I-1                                   | 2.21                      | 1.98 | 3.22    | 4.63          |
| C-I-2                                   | 1.64                      | 1.71 | 2.59    | 3.58          |
| C-I-4                                   | 1.32                      | 1.50 | 2.38    | 2.59          |
| C-I-13                                  | 1.27                      | 1.66 | 2.00    | 2.21          |
| D-II/III/IV-1                           | 1.68                      | 1.84 | 3.15    | 3.91          |
| E-II-1                                  | 2.91                      | 2.90 | 3.70    | 4.68          |
| E-III-1                                 | 1.07                      | 1.45 | 1.66    | 1.47          |
| F-II-2                                  | 1.07                      | 1.22 | 1.65    | 2.33          |
| F-II-3                                  | 0.98                      | 1.24 | 1.40    | 1.60          |
| F-III-1                                 | 1.00                      | 1.32 | 1.80    | 1.59          |
| F-IV-1                                  | 1.29                      | 1.33 | 2.17    | 3.28          |
| F-I-2                                   | 1.27                      | 1.45 | 2.73    | 3.28          |

\(^a\) Key to fraction designation: X = ammonium sulfate cut A–F (see “Experimental Procedures”); Y = AIEX fraction (13 sequential elution fractions, Roman numerals I–XIII); Z = RP HPLC fraction (sequential elutions 1–15).

\(^b\) Disease state-averaged NAvSNR for the fraction/disease state-averaged NAvSNR for all 960 fractions.

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**Fig. 5.** Antibody- and MS-based identification of antigens composing reactive fractions. A, Western blot analyses of fraction C-II-11 with mAb IT-27, pooled cavitary TB patients' sera, and pooled PPD+ healthy control patients' sera (left to right) demonstrate the 38-kDa PstS1 protein as the dominant antigen in this fraction. B, fraction B-III-4 was analyzed (left to right) by SDS-PAGE, Coomassie staining, and Western blot with pooled sera from cavitary TB, noncavitary TB, and healthy PPD+ individuals to demonstrate a 28-kDa protein as the dominant antigen. C, peptide sequences resolved by MS/MS of the Coomassie-stained 28-kDa protein band after trypsin digestion identified the antigen as SodC. AA, amino acid; CAV, cavitary; NON-CAV, noncavitary.
cavitary TB sera. MS/MS analysis of the 20-kDa reactive product in fraction A-VIII-1 resulted in 37% amino acid coverage of the predicted protein sequence encoded by ORF rv3841, and analysis of the silver-stained polyacrylamide gels revealed that the 20-kDa band was the sole protein constituent in both fractions.

TrxC (Rv3914), a 12-kDa thioredoxin (31), was found along with the 45-kDa Apa antigen to account for the serological reactivity of fraction B-II-9. In contrast to the other novel antigen-containing fractions, fraction B-II-9 was only significantly recognized by cavitary TB patients' sera (six of 11).

### Table II

Previously characterized antigens contained in serologically reactive fractions

| Antigen(s)          | Fractionation conditions (X,Y,Z)
|---------------------|-----------------------------------
| 38-kDa PstS1/Rv0934c<sup>a</sup> | C-II-11 1/10 (10%) 1/9 (11%) 6/11 (55%) |
| 45-kDa Apa/ModD/Rv1860<sup>d</sup> | A-II-11 2/10 (20%) 4/9 (44%) 4/11 (36%) |
|                    | A-III-9 2/10 (20%) 4/9 (44%) 5/11 (44%) |
|                    | A-III-11 2/10 (20%) 4/9 (44%) 7/11 (64%) |
|                    | B-III-10 2/10 (20%) 4/9 (44%) 4/11 (36%) |
|                    | B-VI-13 3/10 (30%) 6/9 (67%) 6/11 (55%) |
|                    | B-VI-14 3/10 (30%) 3/9 (33%) 7/11 (64%) |
|                    | A-VI-15 2/10 (20%) 4/9 (44%) 5/11 (44%) |
| Ag85B/Rv1886c<sup>e</sup> | A-VI-3 3/10 (30%) 4/9 (44%) 6/11 (55%) |
|                    | A-VI-4 2/10 (20%) 4/9 (44%) 6/11 (55%) |
|                    | A-VII-3 3/10 (30%) 4/9 (44%) 6/11 (55%) |
|                    | A-VIII-3 2/10 (20%) 4/9 (44%) 6/11 (55%) |
|                    | A-VIII-4 2/10 (20%) 4/9 (44%) 5/11 (44%) |
| GlcB/Rv1837c<sup>c</sup> | A-III-4 2/10 (10%) 4/9 (44%) 6/11 (55%) |
|                    | A-IV-1 1/10 (10%) 5/9 (56%) 6/11 (55%) |
|                    | A-IV-3 2/10 (20%) 4/9 (44%) 6/11 (55%) |
| LAM<sup>c</sup> | A-XI-1 3/10 (30%) 5/9 (56%) 6/11 (55%) |
|                    | A-XI-1 3/10 (30%) 5/9 (56%) 6/11 (55%) |
|                    | A-XI-2 3/10 (30%) 5/9 (56%) 6/11 (55%) |
|                    | B-I-2 2/10 (20%) 4/9 (44%) 6/11 (55%) |
|                    | B-II-2 1/10 (10%) 4/9 (44%) 6/11 (55%) |
|                    | B-III-7 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | B-IV-1 0/10 (0%) 3/9 (33%) 7/11 (64%) |
|                    | C-I-1 0/10 (0%) 1/9 (11%) 6/11 (55%) |
|                    | C-I-2 0/10 (0%) 3/9 (33%) 6/11 (55%) |
|                    | C-I-3 0/10 (0%) 3/9 (33%) 6/11 (55%) |
|                    | C-I-13 2/10 (20%) 3/9 (33%) 5/11 (55%) |
|                    | C-III-1 2/10 (20%) 4/9 (44%) 6/11 (55%) |
|                    | C-IV-1 2/10 (20%) 4/9 (44%) 6/11 (55%) |
|                    | D-III/IV-1 2/10 (20%) 4/9 (44%) 6/11 (55%) |
|                    | E-I-1 0/10 (0%) 3/9 (33%) 6/11 (55%) |
|                    | E-I-3 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | F-I-1 0/10 (0%) 4/9 (44%) 7/11 (64%) |
|                    | F-I-2 2/10 (20%) 5/9 (56%) 6/11 (55%) |
|                    | F-II-2 2/10 (20%) 4/9 (44%) 7/11 (64%) |
| Rv3881c<sup>e</sup> | F-III-3 2/10 (20%) 4/9 (44%) 7/11 (64%) |
|                    | F-IV-1 2/10 (20%) 3/9 (33%) 7/11 (64%) |
|                    | A-VII-10 2/10 (20%) 3/9 (33%) 7/11 (64%) |
| SecE2/Rv0379<sup>e</sup> | A-VII-11 2/10 (20%) 4/9 (44%) 7/11 (64%) |
|                    | A-I-4 2/10 (20%) 4/9 (44%) 5/11 (44%) |
|                    | A-I-5 2/10 (20%) 4/9 (44%) 5/11 (44%) |
|                    | F-I-3 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | A-I-3 1/10 (10%) 4/9 (44%) 6/11 (55%) |
|                    | B-I-4 3/10 (30%) 5/9 (56%) 5/11 (44%) |
| Ag85A/Rv3804c and Ag85B/Rv1886c<sup>e</sup> | F-I-3 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | A-I-1 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | A-I-13 2/10 (20%) 4/9 (44%) 5/11 (44%) |
|                    | A-I-1 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | A-I-13 2/10 (20%) 4/9 (44%) 5/11 (44%) |
|                    | 45 kDa and MPT64/Rv1980c<sup>d</sup> | A-III-10 2/10 (20%) 3/9 (33%) 6/11 (55%) |
|                    | B-I-7 2/10 (20%) 4/9 (44%) 7/11 (64%) |
|                    | C-I-14 2/10 (20%) 3/9 (44%) 6/11 (55%) |

- **a** Key to fraction designation: X = ammonium sulfate cut A–F (see “Experimental Procedures”); Y = AEX fraction (13 sequential elution fractions, Roman numerals I–XIII); Z = RP HPLC fraction (sequential elutions 1–15).
- **b** Number (percentage) of patient sera significantly reactive against fraction in question. Significant reactivity is >3× S.D. above PPD+ mean.
- **c** Antigen identification based on reactivity to an antigen-specific mAb.
- **d** Antigen identification based on reactivity to an antigen-specific mAb and MS/MS analyses.
- **e** Antigen identification based on MS/MS analyses. Detailed MS/MS results are provided in Supplemental Table S1.
acid coverage of the predicted protein sequence encoded by ORF rv3914. Whether or not the significant reactivity of fraction B-II-9 was due to TrxC or the strongly seroreactive 45-kDa antigen (6) could not be determined.

When serological data were combined for those fractions containing the four novel antigens it was found that 56 and 91% of the noncavitary and cavitary TB patients’ sera, respectively, showed positive reactivity. In comparison, when the data were combined for all fractions containing novel and previously identified protein antigens, 78% of noncavitary and 91% of cavitary patients displayed reactivity.

Of considerable interest were the 15 fractions that demonstrated significant reactivity with only HIV+TB+ patients’ sera. Western blot analysis of these fractions with HIV+TB+ patients’ sera failed to demonstrate reactive bands. Furthermore there were no protein bands in common between these fractions when analyzed by SDS-PAGE and silver staining, but treatment of these fractions with Pronase (10 μg/ml for 60 min) prior to microarray printing significantly abrogated reactivity to patients’ sera. Together these observations suggest that 1) a single protein antigen may not be responsible for the reactivity of these 15 fractions, 2) a common antigen such as a small peptide may be responsible for the reactivity, or 3) the reactivity is due to a non-proteinaceous bacterial product complexed with protein.

**DISCUSSION**

Previous studies from our laboratories used 2-D immunoblotting to characterize the profile of Mtb proteins recognized by TB patients’ sera (5, 10). However, this methodology is not well suited for analysis of large numbers of sera due to problems of reproducibility, difficulty in quantifying the results, and refractivity to high throughput analyses. Protein microarrays offer a means by which a large number of sera can be analyzed not only to identify serologically reactive proteins but to establish antigen recognition profiles based on the state or severity of disease (16). At present a complete recombinant protein library of Mtb does not exist. Therefore, to perform protein microarray studies for TB a novel approach involving a robust fractionation strategy that yielded 960 native protein fractions was utilized. The availability of such arrays allowed us to address differences in the patterns of antigens recognized by individuals exhibiting various forms of TB. Specifically TB patients’ sera recognized a much greater number of protein fractions than did sera of PPD+ healthy individuals, and sera of patients with noncavitary TB recognized only a subset (44%) of the fractions that reacted to sera of patients with advanced cavitary disease. This pattern of reactivity agrees well with our previous results obtained by 2-D Western blot analysis where only three to four CFPs reacted to sera of PPD+ healthy individuals where 12 of the 26 cavitary TB-reactive proteins (46%) were recognized by noncavitary TB patients’ sera (5, 10).

The inclusion of antigen identification into these current studies enabled a more in depth assessment of the overlap between the three disease states (cavitary, noncavitary, and HIV+TB+) studied. Of the 55 fractions recognized by both cavitary- and noncavitary-TB patients’ sera, 11 antigens (LAM, the 45-kDa Apa protein, the 19-kDa LpqH protein, Ag85A, Ag85B, Bfrb, GlcB, LppZ, Rv3881c, SecE2, and SodC) were identified as being serologically dominant. Of particular interest is the fact that all five fractions possessing GlcB as the reactive species were recognized by both noncavitary and cavitary TB patients’ sera, confirming previous reports that this antigen is recognized early in disease progression (5, 6). Our analyses also identified a total of 68 fractions recognized exclusively by cavitary TB patients’ sera.

**TABLE III**

**Novel antigens composing reactive fractions**

| Antigen(s)          | Fractionation conditions (X,Y,Z) | Patient reactivity<sup>b</sup> |
|---------------------|---------------------------------|-------------------------------|
|                     |                                 | HIV+TB+ | Noncavitary TB | Cavitary TB | MS/MS<sup>c</sup> |
| Bfrb/Rv3841         | A-VII-1                         | 3/10 (30%) | 4/9 (44%) | 6/11 (55%) | (37) |
| LppZ/Rv3006         | A-VIII-1                        | 3/10 (30%) | 4/9 (44%) | 6/11 (55%) | (37) |
| SodC/Rv0432         | A-III-5                         | 2/10 (20%) | 3/9 (33%) | 6/11 (55%) | (37) |
| Apa/Rv1860<sup>d</sup> and TrxC/Rv3914 | B-VII-9                          | 2/10 (20%) | 4/9 (44%) | 6/11 (55%) | (29)<sup>e</sup> |

<sup>a</sup> Key to fraction designation: X = ammonium sulfate cut A–F (see “Experimental Procedures”), Y = AIEX fraction (13 sequential elution fractions, Roman numerals I–XIII); “/” denotes pooled fractions; Z = RP HPLC fraction (sequential elutions 1–15).

<sup>b</sup> Number (percentage) of patients’ sera significantly reactive against fraction in question. Significant reactivity is >3 × S.D. above PPD+ mean.

<sup>c</sup> Antigen identification based on LC-ESI-MS/MS analysis. Percent amino acid sequence coverage is in parentheses.

<sup>d</sup> The Apa was identified with the mAb CS-93.

<sup>e</sup> The percent amino acid coverage is for TrxC.
Most of the antigens represented by these fractions (the 45-kDa Apa protein, Ag85B, LppZ, SecE2, SodC, LAM, Rv3881c, and the 19-kDa protein) overlapped with those recognized by both cavitary and noncavitary TB patients' sera. However, four antigens (38-kDa PstS1 protein, HspX, MPT64, and TrxC) were recognized only by cavitary TB patients' sera, thus providing several antigens that may be useful in demarcating cavitary and noncavitary TB patients. The identification of the 38-kDa PstS1 as a cavitary TB-specific antigen concurs with previous reports that this antigen is recognized predominantly by patients with advanced disease (5, 6).

Previous experimental approaches have failed to identify an antigen that distinguishes noncavitary TB patients from individuals with other stages of the disease. In this study four native protein fractions were designated as noncavitary TB-specific based on our selection parameters. A more in-depth inspection of these fractions, however, suggests that they are likely not noncavitary TB-specific because these four fractions were each recognized by four of 11 (36%) cavitary TB patients' sera, just missing the 40% cutoff used to construct the Venn diagram. Additionally the 45-kDa Apa protein and the 19-kDa protein were found to be the antigens responsible for the reactivity in three of these four fractions, and these same antigens were also found in five fractions recognized by sera of cavitary TB patients. Thus, a noncavitary TB-specific antigen remains elusive, and it appears that regardless of the methodology used noncavitary TB patients' sera react with a subset of those antigens recognized by cavitary-TB patients' sera.

Although the overall data and conclusions obtained through these microarray-based studies were consistent with earlier work there were a few discrepancies with studies based on 2-D immunoblots (5, 10). In contrast to the qualitative 2-D immunoblot data the results obtained with microarrays were quantitative. Thus, the selection of reactive antigens or fractions was based on the percentage of sera with a NAVSNR value greater than an experimentally determined cutoff. This led to the exclusion of two antigens previously identified by 2-D immunoblots but that fell outside the criteria set in this study to define significant reactivity. This was most notable with GlcB and its reactivity to HIV+TB+ patients' sera. GlcB was previously shown to react with HIV+TB+ sera in both 2-D immunoblot and ELISA formats (3, 10). However, when the microarray data set was quantified the percentage of HIV+TB+ patients' sera that recognized GlcB-containing fractions was just below the 40% cutoff. Previous work shows that sera from this group of patients react to the same set of CFP as recognized by noncavitary TB patients' sera (10). However, our present work defines fractions containing an unidentifiable antigen as the only material with significant reactivity to HIV+TB+ patients' sera. The exceptionally strong response of these fractions with HIV+TB+ sera likely led to a bias in data analysis for this patient group. A second difference with the 2-D Western blot data was the failure to define MPT51 as a dominant antigen. By 2-D PAGE MPT51 readily separates from LAM and is recognized as a dominant antigen in multiple disease states (10). Probing the native protein array slide with the MPT51-specific mAb (IT52) demonstrated that MPT51 cofractionated with LAM (data not shown). Because LAM-containing fractions were excluded from further protein antigen analyses, MPT51 was not designated as a significant serological antigen. In our previous work there were ~12 protein spots that reacted to patients' sera by 2-D immunoblot but were unidentifiable (10). Although it is possible that some of these 2-D protein spots correspond to the four novel antigens identified in our current studies, it is not possible to draw such conclusions without further analyses.

Over the past 2 decades molecular identification of the dominant B and T cell protein antigens of Mtb was achieved through a myriad of techniques and approaches (2, 32, 33). This current application of protein microarrays to TB serodiagnostics has allowed for identification of four novel antigens previously unnoticed by other methods. Two of these antigens, SodC and LppZ, are believed to undergo post-translational modification. SodC was experimentally shown to be lipid-modified and associated with the bacterial membrane (34). This protein also contains three predicted glycosylation sites, and the 40-amino acid N-terminal fragment of SodC was found to bind concanavalin A when fused to the 19-kDa leader sequence and expressed in Mycobacterium smegmatis (35). A method for predicting Gram-positive lipoprotein motifs has also identified LppZ as a probable lipoprotein (36), and similar to SodC, this protein sequence also contains three predicted glycosylation sites (35). The native and recombinant forms of several Mtb proteins have been directly compared, and it is becoming increasingly clear that recombinant forms often lack modifications or conformational epitopes required for immunological recognition (6, 37, 38). The discovery of SodC and LppZ as B cell antigens adds to the growing list of post-translationally modified antigens of Mtb (39) and illustrates the need for a recombinant protein production system that mimics native protein structures.

One of the other novel B cell antigens described in this work (BfrB) was previously shown by us to be a dominant T cell antigen for mice experimentally infected with Mtb (30), and this current work reveals that BfrB is also antigenic in human disease. Thus, like many other Mtb antigens, BfrB is strongly recognized by both the cellular and humoral arms of the immune system and could be targeted as either a vaccine candidate or diagnostic tool. The final B cell antigen newly identified as a significant serological antigen. In our previous work Nagai et al. (40) as MPT46, a major product of the Mtb culture filtrate. Further work established MPT46 as a thioreductase (31). Although this protein was studied previously, the use of protein array technology has provided the first evidence of its antigenic potential.

Through the work of multiple laboratories greater than 16
M*bt proteins have been identified as potential serodiagnostic antigens, and most of these proteins are associated with the culture filtrate (2). However, despite efforts to enrich for proteins of low abundance, we recognize that a relatively small number of novel antigens were identified in this study. The inclusion of denaturing agents may have further improved protein resolution and increased antigen detection; however, such an approach might also destroy conformational epitopes. An alternative explanation for the low number of novel antigens identified is that the discovery of new serodiagnostic antigens from in vitro grown M*bt H37Rv cytosolic and culture filtrate protein pools is nearly exhausted. Nevertheless the cell envelope protein pool of M. tuberculosis is underexploited for serodiagnostic antigen discovery, and the methodologies described in this work lay a foundation for B cell antigen discovery with this fraction. It is likely that further antigen discovery will also be realized once microarray technology is applied to a complete M*bt recombinant protein library.

Our earlier work on the serological response to M*bt proteins in human disease indicated considerable homogeneity in this response among TB patients (10). The previously accepted dogma of heterogeneity in antigen recognition likely resulted from the differences in the immunological response among the various states of the disease and poor immunological reactivity of M*bt recombinant proteins expressed in E. coli (41). The use of protein arrays has confirmed our previous observations and hypotheses. Moreover the ability to assess antigen recognition profiles between disease states allowed for the identification of several proteins recognized by both cavitary and noncavitary TB patients’ sera and at least four proteins that appear diagnostic of cavitary TB. Thus, from these data it will be possible to develop and assess targeted protein arrays that will not only enable serodiagnosis of TB but also the determination of disease severity.

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