An Immunoproteomic Approach for Identification of Clinical Biomarkers for Monitoring Disease

APPLICATION TO CYSTIC FIBROSIS

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Circulating antibodies can be used to probe protein arrays of body fluids, prepared by two-dimensional gel electrophoresis, for antigenic biomarker detection. However, detected proteins, particularly low abundance antigens, often remain unidentified due to proteome complexity and limiting sample amounts. Using a novel enrichment approach exploiting patient antibodies for isolation of antigenic biomarkers, we demonstrate how immunoproteomic strategies can accelerate biomarker discovery. Application of this approach as a means of identifying biomarkers was demonstrated for cystic fibrosis (CF) lung disease by isolation and identification of inflammatory-associated autoantigens, including myeloperoxidase and calgranulin B from sputum of subjects with CF. The approach was also exploited for isolation of proteins expressed by the Pseudomonas aeruginosa strain PA01. Capture of PA01 antigens using circulating antibodies from CF subjects implicated in vivo expression of Pseudomonas proteins. All CF subjects screened, but not controls, were immunoreactive against immunocaptured Pseudomonas proteins, representing stress (GroES and ferric iron-binding protein HitA), immunosuppressive (thioredoxin), and alginate synthetase pathway (nucleoside-diphosphate kinase) proteins, implicating their clinical relevance as biomarkers of infection. Molecular & Cellular Proteomics 4:1052–1060, 2005.

Disease-associated phenotypes are ultimately due to aberrant protein expression or abnormal modification of proteins. Differentially expressed proteins or protein modifications as a consequence of disease and/or disease progression represent biomarker candidates and can be identified using 2-DE-based proteomic approaches (1, 2). Diagnostic/prognostic test formats would ideally monitor changes in biomarker appearance in body fluids, such as sputum, saliva, plasma, or urine, that can be obtained by non-invasive means. Detection of putative biomarkers, particularly low abundance proteins, by protein macroarray profile analysis of complex body fluids is difficult given the large dynamic range of constituent protein levels. Even with sophisticated proteomic methodologies that incorporate a combination of sample preparation techniques, including depletion of high abundance proteins and/or pre-fractionation strategies, detection of protein biomarkers remains challenging (3, 4).

The use of circulating antibody repertoires from subjects suffering diseases such as inflammatory diseases, certain cancers, and autoimmune disorders represents a powerful means for detecting disease-associated antigenic proteins in protein macroarrays derived from body fluids, cell lines, tissue cells, or pathogens, which may be of prognostic/diagnostic value or may represent valuable drug targets (5–8). Despite detection of disease-related antigenic proteins using immunoproteomic approaches (9), many of these antigens remain unidentified due to their low abundance (10, 11). Antigenic biomarker discovery has therefore primarily exploited disease-derived antibody repertoires against random synthetic peptide or cDNA libraries (12, 13), which is both time- and labor-consuming and does not allow detection of antigenic post-translational modifications that are disease-associated.

New methods for enrichment are required for improving detection and identification of disease-related immunogenic proteins in complex protein mixtures to further facilitate clinical biomarker discovery. We addressed two common bottlenecks in clinical antigenic biomarker discovery in this study: (i) detection and identification of disease-related antigens that may be of low abundance and (ii) rapid validation of their immunogenic prevalence to determine their biomarker potential. We present an alternative biomarker discovery strategy (Fig. 1) involving an immunocapture method where circulating antibody repertoires from plasma of patients suffering the same disease are immobilized and subsequently used to capture antigens from complex proteomes such as sputum or
bacterial extracts. Captured antigens are separated by 2-DE to resolve protein isoforms for multiple analyses of each captured protein, including MS-based identification and characterization and validation of their immunogenic prevalence across patient plasma samples by using the chemical inkjet printer ChIP. PTM, post-translational modification.

We demonstrate application of an immunoproteomic strategy to identify a panel of antigenic biomarkers for pulmonary exacerbation in cystic fibrosis (CF). Cystic fibrosis is an infection/inflammation-associated pulmonary disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR)-encoding gene (15). Pathogenic infection of CF lungs from an early stage in life is inevitable and for reasons still unknown ultimately leads to pulmonary bacterial colonization due to inefficient eradication of pathogens, particularly Pseudomonas aeruginosa and Staphylococcus aureus (16). Recurrent exacerbations consequently lead to progressive lung deterioration, which is the major cause of morbidity and mortality in CF subjects. Monitoring the expression of biomarkers that are predictive of pulmonary exacerbation and/or infection would potentially help prolong the survival of CF subjects by helping to dictate short and long term therapy to help minimize cumulative lung damage as a consequence of these cycles of infection and inflammation.

MATERIALS AND METHODS

All chemicals were obtained from Sigma unless otherwise stated. P. aeruginosa PA01 was purchased from ATCC, and cultures were kindly provided by BioTechnology Frontiers Pty. Ltd. (Sydney, New South Wales, Australia).

Clinical Samples—Saline-induced sputum was essentially collected and liquefied as described previously (17) in the presence of protease inhibitors (Roche Applied Science) from healthy control adults (18–45 years old) with forced expiratory volume in 1 s (FEV1), percentage predicted >80%, and from CF adults with an acute pulmonary exacerbation (18, 19), FEV1 < 60%, and requiring hospitalization. Microbiological testing showed profuse pulmonary P. aeruginosa in all CF subjects at the day of hospitalization. These subjects also had a history of additional two to four exacerbations over the previous 12 months. All CF subjects had at least one copy of the ΔF508 mutation. A total of ~7 mg of protein from pooled liquefied sputum samples was used for immunocapture.

Antibody Screening of Protein Macroarrays—The CHAPS-containing ProteomIQTM resuspension reagent (Proteome Systems Inc., Woburn, MA) was used for crude P. aeruginosa protein extractions and to resolubilize acetone-precipitated sputum proteins. Proteins were arrayed by 2-DE. First and second dimension protein separations and protein electroblotting were all conducted on an ElectrophoretIQ3 unit (Proteome Systems Ltd., Sydney, New South Wales, Australia) using homogeneous 14% (w/v) or gradient 6–15% (w/v) polyacrylamide Tris-acetate GelchipTM gels (14). IPG strips were rehydrated with a total of 300 μg of protein extract. After electrotransfer of protein onto PVDF-P membranes (Millipore), each of the membranes was treated with one crude plasma patient sample, diluted to 1.5–2.0 μg/ml, as the primary antibody followed by sheep anti-human IgG as secondary antibody, 1:100,000 dilution (Chemicon Australia Pty., Ltd., Victoria, Australia). Chemiluminescence signals were generated using a SuperSignal ELISA Femto Maximum Sensitivity kit (Pierce).

Western Blot Analysis of Sputum—Five microliters of liquefied sputum were diluted 1:1 (v/v) in Laemmli buffer and boiled for 5 min prior to loading onto 6–15% (w/v) polyacrylamide Tris-acetate one-dimensional gels (Proteome Systems Inc.). Protein electroblotting was conducted on an ElectrophoretIQ3 unit (Proteome Systems Ltd.) using PVDF-P membranes (Millipore). Resulting membranes were each treated with one of the following primary antibodies (all from Silenus Laboratories Pty. Ltd., Melbourne, Victoria, Australia): goat anti-human calgranulin B (diluted 1:2,000), mouse anti-human myeloperoxidase (diluted 1:2,000), and sheep anti-human IgG (diluted 1:150,000). Respective secondary antibodies (diluted 1:150,000) were also from Silenus Laboratories Pty. Ltd. Chemiluminescence signals were generated using a SuperSignal ELISA Femto Maximum Sensitivity kit (Pierce).

Native Protein Extraction—Overnight cultures of P. aeruginosa PA01 (200 ml) were pelleted by centrifugation (4000 × g for 20 min). Cells were washed twice in water and resuspended in 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 20% (w/v) sucrose in the presence of protease inhibitors (Roche Applied Science). Cells were lysed by ultrasonication. Resulting cell debris were removed by centrifugation (4000 × g for 5 min). Cytoplasmic proteins were isolated by centrifugation (40,000 × g for 20 min at 4 °C), and a 1:10 volume of 99% (v/v) acetonitrile was added to the resulting supernatant. Precipitated proteins were isolated by centrifugation and resolubilized in PBS. Membrane proteins were extracted from the pellets generated from the ultrasonication and ultracentrifugation steps using a ProteoPrep membrane extraction kit (Proteome Systems Inc.). Briefly, pellets were resuspended in 100 mM sodium carbonate, and the sample was sonicated for 15 s, placed on ice for 1 min, and resonicated. This was repeated four times followed by a short centrifugation after which the
sonication treatment was repeated on the resulting pellet. The two resulting supernatants were pooled, and the sample was stirred on ice for 1 h. Cell debris was subsequently isolated by centrifugation (5000 × g for 10 min) and discarded. Membrane proteins were isolated by ultracentrifugation (115,000 × g for 75 min), and pelleted proteins were washed twice in 50 mM Tris-HCl, pH 7.3, and resolubilized in PBS containing 1% (v/v) Triton X-100, 15 mM Tris-HCl, pH 7.5, and 20 mM DTT. The sample was subsequently incubated with 60 mM iodoacetamide for 2 h at room temperature to inactive residual DTT. A total of 2.4 mg of protein was retrieved from the two extraction methods.

Isolation of CF-related Antigens—IgG found in 5 ml of crude plasma from four CF subjects with an exacerbation was immobilized onto 5 ml of Protein G-Sepharose (Amersham Biosciences) beads using disuccinimidyl suberate (Pierce) as recommended by the manufacturer and hereinafter referred to as an immunocapture column. Protein G-Sepharose was also used to remove IgG from liquefied sputum samples prior to immunocapture.

The immunocapture column was used to isolate antigenic proteins from native protein extracts by three successive overnight incubations at 4 °C with constant rotation. Briefly the immunocapture beads were pelleted and subsequently resuspended in native protein extracts. After an overnight incubation, the immunocapture column was washed three times with PBS, and captured antigenic proteins were eluted with 50 mM glycine, pH 2.7. Eluted proteins were precipitated and resolubilized using the CHAPS-containing ProteomIQ Resuspension Reagent (Proteome Systems Inc.). Lastly, proteins were arrayed by 2-DE as described above.

MS Analysis—Aliquots of 2-DE-arrayed protein spots were excised, proteolytically digested with 0.05 μg of trypsin (Promega Corp., Sydney, New South Wales Australia) in 50 mM NH₄HCO₃ for 3 h at 37 °C, and the generated peptides were purified as described previously (20, 21). All solutions used for MS analysis were from a ProteomIQ Xcite in-gel digest kit (Proteome Systems Inc.). Peptide mass fingerprints were generated by MALDI-TOF MS using an Axima CFR mass spectrometer (Kratos, Manchester, UK) or an ABI 4700 MALDI MS/MS system (Applied Biosystems). All spectra underwent an internal two-point calibration using autodigested trypsin peak masses, m/z 842.51 and 2211.10 Da. All spectra were converted into ASCII format before submission to an in-house Peak Harvester software tool (Proteome Systems Ltd.), which was used to resolve isotopic peaks from MS spectra and to determine monoisotopic masses (22).

For protein identification purposes, the mass list of peptides was searched against the following data bases of theoretically trypsin-digested “Homo sapiens,” “Other Proteobacteria” in Swiss-Prot/TrEMBL (Swiss-Prot release 43, approximately 160,000 entries), or NCBI’s using the externally available Mascot (www.matrixscience.com) and ProFound (prowl.rockefeller.edu/profound_bin/WebProFound.exe) software. Peptide mass fingerprinting search parameters were as follows: 100 ppm mass accuracy, a single tryptic miss cleavage allowed, propionamide modification of cysteine residues, and a non-fixed oxidation modification of methionine residues. Only significant hits, as defined by the Mascot or ProFound probability analyses, were accepted. Hits with less than four matched peptide masses were only accepted if also confirmed by MS/MS (ABI 4700 MALDI MS/MS) or by PSD analysis (Axima CFR mass spectrometer). Amino acid sequences obtained by PSD analysis were on sulfonated trypsin-digested protein samples (23). MS/MS data base searching was against the Swiss-Prot/TrEMBL or NCBI’s data bases using Mascot software (www.matrixscience.com) with the same search parameters as above but including ion fragment mass accuracies of 0.8 Da. PSD data were analyzed by comparing experimentally obtained ions with a theoretical ion calculation from the parent ion sequence using MS Product from ProteinProspector (128.40.158.151/mshome3.4.htm).

RESULTS

CF-specific Antibody Repertoires—Previous studies imply that an aberrant immune system in CF patients may play a pathological role in CF disease progression (24, 25). The existence of CF-specific antibody repertoires was demonstrated here by screening circulating antibodies from crude plasma of CF subjects with acute pulmonary exacerbations (Fig. 2A) and healthy control subjects (Fig. 2B) against 2-D arrays of sputum proteins derived from a CF patient with an acute exacerbation. The detected antigenic CF-derived sputum proteins (Fig. 2A) were considered biomarkers of exacerbation as they were differentially expressed in sputum from control subjects (Fig. 2C).

All CF subjects used in this study had an acute exacerbation and moderate to profuse P. aeruginosa in sputum at the time of sample collection in addition to two to four pulmonary exacerbations with P. aeruginosa-triggered inflammation over the previous 12 months. We therefore anticipated the presence of P. aeruginosa antibodies in these CF subjects. This was confirmed by screening plasma from CF subjects against a 2-D protein macroarray of a combined cytosolic/membrane P. aeruginosa protein extract (Fig. 2D). In contrast, control subjects showed very low immunoreactivity toward the arrayed P. aeruginosa proteins (Fig. 2E). The detected antigenic P. aeruginosa proteins were considered candidate biomarkers of infection in CF patients.
The vast majority of CF disease-related antigens found in sputum and in *P. aeruginosa* protein extracts, detectable by Western blotting chemiluminescence, could not be detected by staining corresponding 2-D gel arrays due to the complexity of the screened proteomic profiles (Fig. 2F). An alternative method was therefore developed for enrichment and isolation of CF-related antigenic proteins to facilitate MS-based identification.

Fig. 2. Existence of CF-specific antibodies. Circulating antibodies from four CF subjects with acute exacerbations (A) and four control subjects (B) were screened against 2-D macroarrays of proteins isolated from sputum from a CF subject with an acute exacerbation in which representative results are presented here. Circulating antibodies from two of the four CF subjects were also screened against 2-D macroarrays of proteins isolated from sputum from a control subject (C) or 2-D macroarrays of proteins isolated from a combined cytosolic/membrane protein extract from PA01 *P. aeruginosa* (D). Likewise, antibodies from two controls were screened against 2-D macroarrays of PA01 *P. aeruginosa* proteins (E). Control subjects were found to be non-immunoreactive to cognate sputum (data not shown). F, silver-stained 2-DE gel array of CF-derived sputum proteins. Arrows indicate respective anchor points used to orientate the antibody screening data onto replicate 2-DE gel array (marked by asterisks in A and B). Each experiment was repeated twice.

Fig. 3. Isolation of CF-related antigenic proteins. Plasma samples from four CF subjects with an acute exacerbation were pooled, and their circulating antibodies were used to capture immunogenic proteins found in liquefied sputum pooled from two CF subjects with an exacerbation (A) or in non-denatured PA01 *P. aeruginosa* protein extracts (B). Captured proteins were arrayed on pH 3–10 homogeneous 14% 2-DE Gelchip gels (left panel) or 6–15% gradient 2-DE Gelchip gels (right panel) and silver-stained. Aliquots of arrayed proteins were used for MS-based protein identification, and these data are summarized in Tables I and II. Arrows in the left margin indicate full-length immunoglobulin heavy and κ/λ light chains, which were only identified in the 2-DE analysis of captured proteins from sputum. No proteins were captured from liquefied sputum from control subjects incubated with plasma-derived antibodies from CF subjects (data not shown).

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Isolation and Characterization of CF-specific Antigens—Circulating antibodies, isolated from plasma of four CF adults with acute exacerbations, were used to isolate antigenic proteins from both liquefied sputum pooled from two other CF adults with acute exacerbations as well as *P. aeruginosa* PA01 protein extracts. Captured antigens were subsequently separated by 2-DE (Fig. 3). Sections of the protein gel spots were excised and used for MS analysis. Identified antigenic
sputum proteins and *P. aeruginosa* proteins are summarized in Tables I and II, respectively. Capture of PA01 proteins using antibody repertoires from CF patients indirectly implicated that the identified proteins were at some point expressed in *vivo* by clinical strains of *P. aeruginosa*.

Strikingly different antigenic protein profiles were obtained when screening CF antibody repertoires against either denatured (Fig. 2) or non-denatured protein extracts (Fig. 3). The data indicated that the majority of antibodies derived from CF subjects were against epitopes that were only “accessible” in denatured proteins. *In vivo* it is therefore likely that the humoral response triggered by these proteins is due to their uptake by antigen-presenting cells in which epitopes are unmasked as a consequence of protein unfolding and/or degradation.

Capture of calgranulin B, myeloperoxidase (MPO), and lung-specific enolase 1B from CF-derived sputum demonstrated proof-of-concept of our antibody-based enrichment strategy. This was supported by conventional Western blotting where sputum samples from healthy control and CF

### Table I

| Spot no. | Protein identification          | Acc. no. | Matching peptides/ % coverage | TpI/Epl | TMW/EMW | Biological role                                                                 |
|----------|---------------------------------|----------|-------------------------------|---------|---------|---------------------------------------------------------------------------------|
| 1        | Calgranulin B                   | P06702   | 2/26                          | 5.7     | 13/15   | Expressed by macrophages in acutely inflamed tissues and in chronic inflammations. Subunit of the CF antigen complex. |
| 2        | Calgranulin B                   | 4/40     | 5.7                           | 13/14   |         |                                                                                  |
| 3        | Calgranulin B                   | 4/39     | 5.7                           | 13/15   |         |                                                                                  |
| 4–5      | Not identified                  |          |                               |         |         |                                                                                  |
| 6        | Ig γ heavy chain fragment        | 4/27     | 8.5                           | 55/15   |         | Central part of the adaptive immune system. Fc region mediates antigen opsonization. |
| 7        | Ig γ heavy chain fragment        | 3/29     | 8.5                           | 55/15   |         |                                                                                  |
| 8        | Ig γ heavy chain fragment        | 5/36     | 8.5                           | 55/15   |         |                                                                                  |
| 9        | Ig γ heavy chain fragment        | 3/12     | 8.5                           | 55/15   |         |                                                                                  |
| 10       | Myeloperoxidase fragment        | P05164   | 6'/15                         | 5.8     | 85'/14  | Host defense component with microbicidal activity against various pathogenic organisms. Classic ANCA target. |
| 11       | Not identified                  |          |                               |         |         |                                                                                  |
| 12       | Lung-specific enolase 1B        | Q05524   | 4/17                          | 7.0     | 47/12   | Glycolytic enzyme. Classic ANCA target.                                          |
| 13       | Not identified                  |          |                               |         |         |                                                                                  |
| 14       | Not identified                  |          |                               |         |         |                                                                                  |

*a* Percent sequence coverage based on the full-length sequence of non-processed myeloperoxidase.

*b* Molecular weight of the non-processed myeloperoxidase.

*c* Anti-neutrophil cytoplasmic antibodies.

### Table II

| Spot no. | Protein identification          | Acc. no. | Matching peptides/ % coverage | TpI/Epl | TMW/EMW | Biological role                                                                 |
|----------|---------------------------------|----------|-------------------------------|---------|---------|---------------------------------------------------------------------------------|
| 1        | Ferric iron-binding protein HitA, fragment | Q9HVA8   | 4/16                          | 5.5     | 36/10   | Putative iron transport protein. Periplasmic location.                          |
| 2        | Thioredoxin-dependent PAPS reductase, fragment | O05927   | 4/14                          | 6.0     | 30/10   | Regulatory role in alginate synthesis                                           |
| 3        | Thioredoxin                     | Q9X2T1   | 6/56                          | 4.7     | 12/12   | Putative stress                                                                 |
| 4        | Thioredoxin                     | Q9X2T1   | 7/67                          | 4.7     | 12/12   | Response protein                                                                |
| 5        | Not identified                  |          |                               |         |         |                                                                                  |
| 6        | GroES                           | P30720   | 9/74                          | 5.2     | 12/12   | Immunodominant heat shock protein                                               |
| 7        | NDK                             | Q59636   | 6/69                          | 5.5     | 16/15   | Regulatory role in alginate synthesis                                           |
| 8        | Not identified                  |          |                               |         |         |                                                                                  |
| 9        | DNA-binding protein HU          | P05384   | 6/64                          | 9.7     | 10/8    | Stress response protein                                                          |
subjects were screened with commercially available antibodies for the appearance of calgranulin B and MPO (Fig. 4A). Calgranulin B is the regulatory subunit of the “CF antigen” (26), and MPO and enolase are known autoantigens in several inflammatory diseases including CF (27–29). Several fragments of the heavy chain of IgG were captured from CF-derived sputum. Subsequent Western blotting analysis of sputum from healthy control and CF subjects confirmed the appearance of Ig γ heavy chain fragments (Fig. 4B). Immunoreactive IgG heavy chain fragments have been observed in certain autoimmune disorders (30) and chronic inflammatory diseases (31). Several protein isoforms of calgranulin B were captured by plasma-derived antibodies isolated from CF subjects. Analysis by MS of the captured antigenic calgranulin B isoforms referred to as spots 1 and 2 in Fig. 3A showed both isoforms were phosphorylated (data not shown), which has been reported previously (32).

Validation of Clinical Immunogenicity of Immunocaptured P. aeruginosa Proteins—Chemical inkjet printing was used to confirm that the four individual CF subjects, used to generate the immunocapture column, were immunoreactive to captured proteins. Serological immunoreactivities of the four CF patients as well as three healthy non-CF controls were simultaneously determined by depositing 0.75–μl quantities of 1:3 diluted sera from each of these individuals onto each of four of the cultured P. aeruginosa PA01 proteins, GroES, thioredoxin, NDK, and ferric iron-binding protein HitA, using a chemical inkjet printer, ChIP (Fig. 5). All CF subjects screened were immunoreactive toward all four screened pathogenic proteins, albeit to various degrees, in contrast to the serologically non-reactive control subjects. These data imply that the identified pathogen-encoded proteins were, or still are, clinically expressed in CF subjects.

**DISCUSSION**

Application of a new immunoproteomic strategy as a means of identifying antigenic proteins associated with disease has been demonstrated by analysis of CF as a respiratory disease. Validation of the concept of the immunocapture technique was confirmed by CF antibody-based isolation of well known chronic inflammation-associated autoantigens, including enolase 1B and myeloperoxidase and the regulatory subunit calgranulin B of the known CF antigen complex (26), from sputum of CF subjects with acute exacerbations. Autoimmune fragments of immunoglobulin γ (IgG) heavy chains were also isolated. IgG fragments as well as autoantibodies against myeloperoxidase and calgranulin B have all previously been suggested to be markers of acute inflammation in CF subjects (27, 33–34).

Development of autoantibodies is common in several infection-associated diseases (25, 35), which may be triggered by exposure of cryptic “self” epitopes due to their increased concentration, ectopic location, extracellular proteolysis, or peptide mimicry (36, 37). Screening of antibodies from CF subjects with acute exacerbations against native sputum proteins, using an immunoffinity-based enrichment strategy, resulted in the isolation of an antigenic 12-kDa enolase 1B fragment (Fig. 3A, spot 12). Full-length enolase 1B was immunologically detected only when screening plasma-derived antibodies from CF subjects against denatured 2-DE sputum protein arrays (data not shown). This data indicated the existence of a cryptic epitope that is exposed in the 12-kDa fragment. Serum autoantibodies against enolase 1 have been reported in a diverse range of inflammatory and degenerative disorders (38), and pathogen-induced exposure of cryptic self-epitope(s) in enolase 1 has been linked previously to development of autoimmunity in infectious diseases other than CF (39, 40). Lack of efficient eradication of opportunistic pathogens in CF subjects, particularly P. aeruginosa, is not understood. It is therefore intriguing that our enrichment strategy resulted in the immunocapture of P. aeruginosa-encoded proteins such as GroES and thioredoxin, which are known immunosuppressive pathogenic factors in other chronic inflammatory diseases (41, 42), as well as the capture of human IgG fragments. Appearance of immunogenic IgG fragments has been linked to pathogen-induced autoimmunity and pathogen persistence in several infectious diseases (31, 43). Bacterial levels can be significantly reduced by antibiotic treatment if detected early in CF subjects. Detection of infec-
tion in lower respiratory tracts is often symptom-driven and frequently based on microbiological screening of deep throat swabs or expectorated sputum or less commonly on antibody titer measurements (44–46). Thus, substantial lung damage may have occurred prior to detection of bacteria within the respiratory system by conventional diagnostic methods.

Initial discovery of pathogen-encoded biomarkers in clinical samples is extremely difficult due to a rapid immune-triggered degradation of pathogen-derived proteins and the dynamic complexity of body fluids. We used plasma-derived antibody repertoires from CF subjects with acute exacerbations to isolate antigenic proteins from the laboratory non-mucoid \( P. \) \textit{aeruginosa} \textit{PA01} strain. Clinical studies have shown that pulmonary \( P. \) \textit{aeruginosa} infections in CF subjects are initially by non-mucoid \( P. \) \textit{aeruginosa} strains that subsequently transform into mucoid alginate-producing variants (44). The immunocaptured bacterial proteins we identified are involved in anaerobic stress response (thioredoxin, ferric iron-binding protein HitA, GroES, and DNA-binding protein HU) and alginate synthesis (NDK and thioredoxin-dependent PAPS reductase) (47–49). Alginate production is a dominant virulence factor of clinical \( P. \) \textit{aeruginosa} strains. Clinical \textit{in vivo} expression was further demonstrated for four of the six immunocaptured proteins (NDK, HitA, thioredoxin, and GroES) where all CF subjects screened were found to be immunoreactive toward these proteins in contrast to control subjects (Fig. 5).

Hence the antibody-based enrichment method described here can expedite identification of clinically relevant low abundance pathogen-derived proteins from laboratory cultures and/or clinical isolates as well as determine patient-to-patient differences in immune responses to a specific biomarker. \( P. \) \textit{aeruginosa} biomarker discovery has primarily involved differential protein expression analyses of laboratory strains and/or clinical isolates grown under various \textit{in vitro} conditions (50, 51), but \textit{in vivo} expression of these identified differentially expressed proteins has yet to be demonstrated. Of the identified proteins in this study, only GroES has been targeted previously in a PCR-based diagnostic test format for prediction of \( P. \) \textit{aeruginosa} infection (52). For this study we used the \( P. \) \textit{aeruginosa} PA01 strain to identify markers of \textit{Pseudomonas} infection. Analysis of additional laboratory and clinical isolates will undoubtedly reveal further proteins of interest. Determining the clinical expression levels of identified \( P. \) \textit{aeruginosa} proteins in CF subjects pre- and post-\( P. \) \textit{aeruginosa} infections as well as in chronically infected CF patients with acute exacerbations will help enable development of a point-of-care diagnostic test format to monitor onset of exacerbation as well as monitor disease progression in CF patients in a non-invasive manner.

We anticipate that the presented immunoproteomic strategy will facilitate rapid deciphering of targets to further validate in clinical trials without requiring prior gene cloning,
protein expression and purification, and antibody generation. The presented immunocapture method is an important tool for clinical proteomic biomarker discovery as it can expand the repertoire of biomarkers available for development of point-of-care tests for rapid and accurate diagnosis and prognosis of disease.

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