Immunoproteomic Analysis of Antibody in Lymphocyte Supernatant in Patients with Typhoid Fever in Bangladesh

Richelle C. Charles, Li Liang, Farhana Khanam, M. Abu Sayeed, Chris Hung, Daniel T. Leung, Stephen Baker, Albrecht Ludwig, Jason B. Harris, Regina C. LaRocque, Stephen B. Calderwood, Firdausi Qadri, Philip L. Felgner, Edward T. Ryan

Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts, USA; Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA; Department of Medicine, University of California, Irvine, California, USA; International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), Dhaka, Bangladesh; Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam; Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Klinikum der Johann Wolfgang Goethe-Universitat, Frankfurt am Main, Germany; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA; Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA; Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, USA.

We have previously shown that an assay based on detection of anti-Salmonella enterica serotype Typhi antibodies in supernatant of lymphocytes harvested from patients presenting with typhoid fever (antibody in lymphocyte supernatant [ALS] assay) can identify 100% of patients with blood culture-confirmed typhoid fever in Bangladesh. In order to define immunodominant proteins within the S. Typhi membrane preparation used as antigen in these prior studies and to identify potential biomarkers unique to S. Typhi bacteremic patients, we probed microarrays containing 2,724 S. Typhi proteins with ALS collected at the time of clinical presentation from 10 Bangladeshi with acute typhoid fever. We identified 62 immunoreactive antigens when evaluating both the IgG and IgA responses. Immune responses to 10 of these antigens discriminated between individuals with acute typhoid infection and healthy control individuals from areas where typhoid infection is endemic, as well as Bangladeshi patients presenting with fever who were subsequently confirmed to have a nontyphoid illness. Using an ALS enzyme-linked immunosorbent assay (ELISA) format and purified antigen, we then confirmed that immune responses against the antigen with the highest immunoreactivity (hemolysin E [HlyE]) correctly identified individuals with acute typhoid or paratyphoid fever in Dhaka, Bangladesh. These observations suggest that purified antigens could be used with ALS and corresponding acute-phase activated B lymphocytes in diagnostic platforms to identify acutely infected patients, even in areas where enteric fever is endemic.

Enteric fever is caused by the human-restricted pathogens Salmonella enterica serotype Typhi, serotype Paratyphi A, and rarely by serotype Paratyphi B. Enteric fever is endemic throughout the African and Asian continents, with typhoid fever causing an estimated 21.7 million cases per year and over 200,000 deaths annually and paratyphoid fever causing over 5 million illnesses per year. Clinical symptoms of enteric fever correlate with bacteremia and include fevers, malaise, and abdominal pain, with potential complications, including encephalopathy and intestinal perforation. Accurate diagnosis requires laboratory confirmation; unfortunately, there are currently no reliable diagnostic assays for enteric fever. A reliable assay for enteric fever is needed not only for the diagnosis of acute infection but also for use in surveillance programs to assess disease burden within a community and evaluate prevention programs. Currently available diagnostics for enteric fever include blood culture that is positive in only 40 to 60% of presumptive cases. Bone marrow culture, the gold standard for diagnosis, has improved sensitivity, but its use is limited due to technical challenges and its invasiveness. Antibody detection assays such as the Widal assay, which detects agglutinating antibody responses to S. Typhi lipopolysaccharide (LPS) (O) antigen and flagellar (H) antigen, are often nonspecifically cross-reactive in areas where enteric fever is endemic and ideally require comparison of responses in acute-phase versus convalescent-phase serum samples. Other commercially available antibody-based serological assays have similar limitations with sensitivities and specificities in areas where typhoid fever is endemic. Typhoidal Salmonella bacteria cause minimal intestinal inflammation but survive within professional phagocytic cells, circulate systemically, and lead to a systemic state of inflammation. Activated lymphocytes, induced by a range of pathogens, are detectable in peripheral blood early in infection and can be evaluated for antigen-specific responses. These cells can be cultured ex vivo without specific antigenic stimulation. During culturing, these already activated lymphocytes secrete antigen-specific antibodies into the culture supernatant that can then be detected via an enzyme-linked immunosorbent assay (ELISA). Such liquid-based assays have been referred to...
as ALS (antibody in lymphocyte supernatant)-based assays, and their use has been described after infection or vaccination for a number of pathogens, including the pathogens causing cholera (11, 13), tuberculosis (14), typhoid fever (10, 15, 16), and influenza (17).

We have previously shown that an ALS assay based on detection of anti-S. Typhi antibodies in the supernatant of activated lymphocytes harvested from patients with acute-phase typhoid fever can identify 100% of patients with blood culture–confirmed typhoid fever and paratyphoid A fever in Bangladesh (10, 16). This initial assay was developed using a crude membrane preparation of S. Typhi as the target antigen. Therefore, we aimed to define immunodominant antigens within this membrane preparation and to identify potential biomarkers unique to S. Typhi bacteremic patients that can be used in an ALS-based assay.

MATERIALS AND METHODS

Study subject selection and sample collection. Individuals presenting to the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) hospital or the Kamalapur field site of the icddr,b with possible typhoid fever were eligible for enrollment if they met the following criteria at presentation: age of 1 to 59 years, fever duration of 3 to 7 days (≥39°C), no obvious focus of infection, and no alternate diagnosis. We collected 5 to 10 ml of venous blood from participants when they were enrolled in the study (day 0). Three to five milliliters of blood was used for microbiologic analysis using a BacT/Alert automated system. We subcultured bottles flagged as positive on MacConkey agar and identified Salmonella enterica serotype Typhi isolates in blood samples from 25 individuals using standard biochemical tests and reaction with Salmonella-specific antisera (18).

After we collected the initial blood samples, we treated patients with parenteral ceftaxime or oral cefixime for up to 14 days at the discretion of the attending physician. We also collected venous blood samples from 9 individuals with S. Paratyphi A bacteremia, 5 individuals with other febrile illnesses based on clinical findings and laboratory tests (i.e., 2 patients with visceral leishmaniasis confirmed by PCR and 3 patients with tuberculosis confirmed by a positive acid-fast bacillus sputum smear), and 10 healthy Bangladeshis. These studies were approved by the human studies committees of the icddr,b, Massachusetts General Hospital, and the University of California, Irvine.

Collection of antibody in lymphocyte supernatants. We generated ALS from Bangladeshi patients as previously described (10). Briefly, we diluted heparinized venous blood 1:2 in phosphate-buffered saline and used density gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) to isolate peripheral blood mononuclear cells (PBMCs). We resuspended cells to a concentration of 107 cells/ml in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Ogden, UT), 200 mM β-glutamine, 100 mM pyruvate, 100 U of penicillin/ml, and 100 μg of streptomycin/ml and incubated PBMCs at 37°C in 5% CO2. After 48 h of incubation, we collected the culture supernatants, added a protease inhibitor, and stored samples frozen at −80°C.

Microarray construction and probing. Protein arrays containing 2,724 S. Typhi antigens (63% of proteome) were constructed as previously described (19). Proteins included in arrays were selected based on features enriched in seroreactive antigens in other bacterial species (e.g., proteins with signal peptide motifs and/or motifs characteristic of lipoproteins or proteins associated with the outer membrane or periplasm, as well as heat shock, chaperone, transport, or virulence proteins). Proteins that were nonhomologous (<50% identity at the amino acid level) to Escherichia coli were also included. The arrays were probed with ALS of 10 patients with confirmed S. Typhi bacteremia, 5 healthy Bangladeshis, and 5 patients with other febrile illnesses. The arrays were probed with ALS fluid diluted 1:2 with protein array blocking buffer (Schleicher & Schuell, Keene, NH) supplemented with E. coli (DH5α) lysose (McLab, San Francisco, CA) at a final concentration of 1 mg/ml protein to block anti-E. coli antibodies. Bound antibody was detected with biotin-conjugated anti-human IgG or IgA secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:400 in blocking buffer, followed by streptavidin conjugated with Streptavidin HRP (Columbia Biosciences, Frederick, MD). The slides were scanned and analyzed using PerkinElmer ScanArray Express (Waltham, MA), and signal intensities were quantified using QuantiArray software (PerkinElmer, Waltham, MA).

Data analysis. Analysis of the protein microarray data was accomplished according to our previously published computational methods (19, 20). Microarray spot intensities were quantified using QuantiArray software utilizing automatic local background subtraction for each spot. “No-DNA” negative controls consisted of in vitro transcription/translation reaction without the addition of plasmid template (19). “No-DNA” spots on each array were averaged, and this negative-control background value was subtracted from every other spot on the array. Variance stabilization and normalization (VSN) was applied to quantified array intensities. VSN normalization was performed using the R statistical programming language and implemented as part of the Bioconductor suite (www.bioconductor.org). In addition to removing heteroscedasticity, this procedure corrects for nonspecific noise effects by finding maximum likelihood shifting and scaling parameters for each array such that control probe variance is minimized (21, 22). Proteins were considered immunoreactive if signal intensity was greater than the average signal intensity plus 2 times the standard deviation of all negative-control “no-DNA” spots. Differentially reactive proteins between infected and uninfected groups were determined using a Nayes regularized t test adapted from Cyber-T for protein arrays (23), which has been shown to be more effective than other differential expression techniques. A P value smaller than 0.05 was considered significant.

Detection of anti-HlyE IgA responses in ALS by ELISA. To validate the results of our microarray immunoscreen, we selected the antigen with the most prominent immunoreactivity, hemolysin E (HlyE, rH1477). Using an ELISA format, we analyzed the immunoreactivity of HlyE in ALS at the time of presentation of 15 individuals with confirmed S. Typhi bacteremia (not included in our above immunoscreen), 9 individuals with confirmed S. Paratyphi A bacteremia, and 10 healthy Bangladeshis controls. We coated plates with 100 ng/well of purified HlyE prepared as previously described (24) and detected antigen–specific responses by adding 100 μl of ALS (diluted 1:2), detected with anti-human IgA conjugated with horse-radish peroxidase at a 1:1,000 dilution, and we measured peroxidase activity with ortho-phenylene diame (Sigma Chemical Co., St. Louis, MO) as previously described (10). We assessed differences between groups using a Mann-Whitney U test, and a P value smaller than 0.05 was considered significant.

RESULTS

ALS IgG and IgA profiles. We probed protein arrays containing 2,724 S. Typhi antigens with ALS from 10 individuals with confirmed S. Typhi bacteremia. In total, we identified 62 immunoreactive S. Typhi proteins as defined as having a mean reactivity greater than 2 times the standard deviation above the mean of the no-DNA controls. Nineteen antigens were specific to IgG, 33 antigens were specific to IgA, and 10 were common to both antibody subclasses (Fig. 1). These antigens included a number of membrane-associated proteins, including a hemolysin, lipoproteins, fimbrial proteins, and transport proteins (see Table S1 in the supplemental material).

Ten antigens (listed below using the S. Typhi Ty2 open reading frame [ORF] label) were identified in our differential immunoscreen using ALS from individuals with acute typhoid infection, healthy Bangladeshis, and Bangladeshi patients presenting with fever who were subsequently confirmed to have a nontyphoid illness. Of the 10 antigens, 7 antigens were found to have significi-
cantly higher IgG immunoreactivity compared to healthy controls and individuals with other febrile illnesses (Cyber-T P value < 0.05): hemolysin E, HlyE (t1477); nonspecific acid phosphatase precursor, PhoN (t4225); toxin-like protein, CdtB (t1111); fimbral subunits SthD (t4631), SthA (t4634), and BcfA (t0022); and an outer membrane protein, OmpS2 (t1341). HlyE, homoprotocatechuate degradative operon repressor (HpcR, t1819), a putative ethanolamine utilization protein, EutN (t0394), and a tail-specific protease, Prc (t1033), had statistically significantly higher IgA immunoreactivity when typhoid patients were compared to healthy controls and those with other febrile illnesses.

**Anti-HlyE IgA responses in ALS.** To further characterize HlyE immunoreactivity in ALS, we evaluated immunoreactivity to purified HlyE using an ELISA-based format and IgA detection. We assessed anti-HlyE responses in ALS at the time of presentation of 15 individuals with confirmed S. Typhi bacteremia (individuals not included in our immunoscreen), 9 individuals with confirmed S. Paratyphi A bacteremia, and 10 healthy controls. We found significantly higher IgA immunoreactivity to HlyE in patients with S. Typhi (P < 0.0001) or S. Paratyphi A fever (P = 0.001) compared to healthy Bangladesis (Fig. 2). All 15 patients with acute typhoid fever (100%) and 8 out of 9 patients with paratyphoid A bacteremia (88.9%) had a significant IgA response to HlyE.

**FIG 1** ALS IgG and IgA profiles. (A and B) S. Typhi arrays probed for IgG (A) and IgA (B) responses with ALS from individuals with acute typhoid infection, healthy Bangladeshis, and Bangladeshi patients presenting with fever who were subsequently confirmed to have a non-typhoid illness. On the heat map, antigens are listed on the vertical axis, and individual ALS samples by cohort are listed along the top. The mean IgG or IgA reactivity to the antigens for each sample cohort is plotted on the graph, and the Cyber-T P values for comparison of acute typhoid patients to healthy controls and other febrile controls are plotted on the right-hand y axis. A cutoff P value of <0.05 was considered significant.
fever (88.9%) had a detectable anti-HlyE IgA response (ELISA unit [EU] > 1) (Fig. 2). None of 10 healthy Bangladeshi (0%) had an anti-HlyE IgA response.

**DISCUSSION**

There is currently no optimal assay for diagnosing patients with acute typhoid or paratyphoid fever or for assessing enteric fever disease burden within a community. Detection of S. Typhi or S. Paratyphi A by microbiologic culture or nucleic acid amplification techniques is limited due to low bacterial burden present in peripheral blood, and serodiagnostic assays in areas where these bacteria are endemic are hampered by the high likelihood of prior exposure to these organisms (2). New alternative approaches for typhoid diagnostic assays and surveillance tools are needed. In this analysis, we took advantage of the rapid expansion of activated antigen-specific antibody-secreting cells (ASC) that occurs following infection or vaccination. These activated lymphocytes can act as an early biomarker that can be used in diagnostic assays, and given their transient nature, they could prove particularly useful in developing assays for use in areas where enteric fever is endemic.

We have previously shown that an assay based on detection of anti-S. Typhi antibodies in supernatant of lymphocytes (ALS) harvested from patients with typhoid fever can identify 100% of patients with blood culture-confirmed typhoid fever and paratyphoid A fever in Bangladesh (10, 16). This initial assay was developed using a crude membrane preparation of S. Typhi as the target antigen (10), and at the present time, it is used as a diagnostic assay at the icddr,b clinical facilities for diagnosis of enteric fever (16). Here we extend these observations by probing S. Typhi protein arrays with ALS of patients with confirmed S. Typhi bacteremia to assess the antigenic profile of antibodies in ALS. We were able to identify 62 immunoreactive antigens in total when evaluating both IgG and IgA. Of these responses, 10 antigens discriminated between individuals with acute typhoid infection and healthy Bangladeshi controls, as well as Bangladeshi patients presenting with fever who were subsequently confirmed to have a nontyphoidal illness. These antigens included HlyE, CdtB, PhoN, SthD, SthA, BcfA, HpcR, Prc, EutN, and OmpS2. Prior studies have shown that individuals with typhoid fever develop strong anti-HlyE, anti-CdtB, and anti-PhoN serum responses (19, 25, 26).

Hemolysin E (HlyE), also referred to as ClyA, had the highest IgG and IgA immunoreactivity in our screen. Hemolysin E is a pore-forming toxin that contributes to the cytotoxicity and invasion of epithelial cells and also affects bacterial growth within human macrophages (27–29). HlyE shares >90% amino acid identity with ClyA in E. coli K-12 (29), and in S. Typhi, it has been shown to be expressed under the control of the PhoP regulon (27, 30) that regulates gene expression important in intracellular survival of S. enterica. Within the genus Salmonella, HlyE was originally found only in the typhoidal serotypes S. Typhi and S. Paratyphi A (29), but an analysis of a larger spectrum of salmonellae (by genome sequencing, comparative genomic hybridization, and PCR analysis) recently revealed that the hlyE gene has a wider distribution and that it is also present in some of the nontyphoidal Salmonella serotypes (including, but not limited to, invasive isolates of Salmonella serotypes Schwarzengrund, Montevideo, Bredeney, and others) (28, 31, 32).

Cytolethal distending toxin (CdtB) is one of the A components of typhoid toxin, a unique AB-type toxin made up of 2 A subunits (CdtB and pertussis-like toxin A [PhtA]), and 5 B subunits (PhtB, a homolog of one of the heterologous B subunits of pertussis toxin). CdtB is a DNase that is upregulated intracellularly and induces cell cycle arrest of host cells by causing DNA damage (33). Typhoid toxin, through its CdtB subunit, is responsible for many of the symptoms associated with acute typhoid infection (34). Like HlyE, CdtB is present in S. Typhi and S. Paratyphi A but is rarely found in other Salmonella serovars (35).

We also identified fimbrial subunits (SthD, SthA, and BcfA) that are unique to Salmonella. The sth and bcf fimbrial operons play a role in colonization and long-term intestinal persistence of S. Typhimurium in a mouse model (36), and antibody responses to BcfA and SthA have been demonstrated after S. Typhimurium infection in mice (37). In a comparative genomic hybridization analysis of invasive nontyphoidal Salmonella isolates from bacteremic human patients, the sth and bcf fimbrial operons were found to be part of a core set of 5 fimbrial operons that are conserved across isolates of invasive nontyphoidal Salmonella (31).

OmpS2 is a porin found in Salmonella spp.; along with major porins such as OmpC and OmpF, it plays a role in virulence in S. Typhimurium (38). In a mouse model, OmpS2 was also found to be a potent inducer of the innate immune response acting as both a Toll-like receptor 2 (TLR2) and TLR4 agonist (39). In addition, OmpS2 has adjuvant capability, boosting antibody responses when coimmunized with other antigens (39).

In our analysis, we also identified PhoP-regulated nonspecific acid phosphatase, PhoN (30); homoprotocatechuate degradative operon repressor, HpcR, a negative regulator of the hpc cluster involved in catabolism of aromatic amino acids (40); and EutN, an ethanolamine utilization protein in the eut operon involved in use of alternative carbon sources. We have previously demonstrated upregulation of the eut operon in individuals bacteremic with S. Typhi and S. Paratyphi A (41, 42). These antigens are common to all Salmonella enterica serotypes and are also found in E. coli and other Gram-negative organisms.

To validate our findings, we assessed IgA ALS responses to purified HlyE in a standard ELISA format. We focused on IgA responses since we had previously shown that ALS IgA responses...
are transient markers of recent infection (10). Using such an approach, we confirmed both a high specificity (100%) and a high sensitivity of 100% and 89% in identifying patients bacteremic with either S. Typhi or S. Paratyphi A, respectively.

Measurement of responses in ALS fluid may have several advantages over assessing responses in serum. We have previously shown that 84% of positive ALS responses become negative by day 21 (the last time point we have evaluated) (10, 16), thus supporting the idea that the ALS response is due to recent acute infection and would be a useful diagnostic in areas where there is a high prevalence of prior exposure to S. Typhi or S. Paratyphi A. In addition, we observed marked differences in immunoreactivity in microarrays using ALS from patients with acute S. Typhi infections compared to ALS from healthy individuals in areas where enteric fever is endemic and febrile Bangladeshi controls. These observations suggest that ALS and acute-phase lymphocytes could be used to identify acutely infected patients, even in areas where enteric fever is endemic.

A limitation of this analysis is that we included only individuals from Bangladesh and had a low number and variety of other febrile illnesses included in our analysis; thus, further testing will be needed to further investigate the specificity of our assay and the generalizability of our findings to other geographic areas where typhoid and paratyphoid fever are endemic. In addition, we performed our microarray immunoprobe using ALS from patients infected with S. Typhi, and it may be useful to identify antigens that would distinguish S. Typhi from S. Paratyphi A infection. Although the ALS assay at this time is not a point-of-care test, we needed to further investigate the specificity of our assay and the generalizability of our findings to other geographic areas where typhoid and paratyphoid fever are endemic. In addition, we performed our microarray immunoprobe using ALS from patients infected with S. Typhi, and it may be useful to identify antigens that would distinguish S. Typhi from S. Paratyphi A infection. Although the ALS assay at this time is not a point-of-care test, we have previously shown that the analysis can be performed in areas with limited laboratory setups and requires minimal training of personnel (16), suggesting it might have particular utility as a surveillance tool. Our identification of a core set of target antigens, and confirmation of assay utility using a purified antigen (HlyE), should also facilitate development of a sensitive, specific, and reproducible assay.

In conclusion, we have performed an immunoscreen of anti-S. Typhi responses present in the supernatant of naturally activated lymphocytes recovered from individuals acutely bacteremic with S. Typhi. Our analysis has revealed a subset of antigens, including HlyE, and immunoreactivity against these antigens was able to distinguish patients with acute typhoid fever from healthy controls and febrile patients with other illnesses. The screening of protein arrays with ALS is a method that could be applied to a number of pathogenetics to identify candidate antigens that could be used in diagnostic assay development.

ACKNOWLEDGMENTS

This work was supported by the icddr,b and grants from the National Institutes of Health, including the National Institute of Allergy and Infectious Diseases (AI100023 and AI077883 [E.T.R. and F.Q.], AI085935 [S.B.C., E.T.R., and F.Q.], AI073672 [L.L. and P.L.F.], AI078213 [L.L. and P.L.F.], Sida [F.Q. and F.K.], Training Grant in Vaccine Development and Public Health (D43 TW005572 [F.K., E.T.R., and F.Q.]), Career Development Awards (K08 AI089721 [R.C.C.], K08 AI100923 [D.T.L.]), a Massachusetts General Hospital Physician Scientist Development Award (R.C.C.), a Burroughs Wellcome Fund/American Society of Tropical Medicine and Hygiene Postdoctoral Fellowship in Tropical Infectious Diseases (D.T.L.), and grants from the Deutsche Forschungsgemeinschaft (LU 842/1-1 [A.L.]).

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