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Salmonella enterica Serovar Typhi-Specific Immunoglobulin A Antibody Responses in Plasma and Antibody in Lymphocyte Supernatant Specimens in Bangladeshi Patients with Suspected Typhoid Fever

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Many currently available diagnostic tests for typhoid fever lack sensitivity and/or specificity, especially in areas of the world where the disease is endemic. In order to identify a diagnostic test that better correlates with typhoid fever, we evaluated immune responses to Salmonella enterica serovar Typhi (serovar Typhi) in individuals with suspected typhoid fever in Dhaka, Bangladesh. We enrolled 112 individuals with suspected typhoid fever, cultured day 0 blood for serovar Typhi organisms, and performed Widal assays on days 0, 5, and 20. We harvested peripheral blood lymphocytes and analyzed antibody levels in supernatants collected on days 0, 5, and 20 (using an antibody-in-lymphocyte-supernatant [ALS] assay), as well as in plasma on these days. We measured ALS reactivity to a serovar Typhi membrane preparation (MP), a formalin-inactivated whole-cell preparation, and serovar Typhi lipopolysaccharide. We measured responses in healthy Bangladeshi, as well as in Bangladeshi febrile patients with confirmed dengue fever or leptospirosis. We categorized suspected typhoid fever individuals into different groups (groups I to V) based on blood culture results, Widal titer, and clinical features. Responses to MP antigen in the immunoglobulin A isotype were detectable at the time of presentation in the plasma of 81% of patients. The ALS assay, however, tested positive in all patients with documented or highly suspicious typhoid, suggesting that such a response could be the basis of improved diagnostic point-of-care assay for serovar Typhi infection. It can be important for use in epidemiological studies, as well as in difficult cases involving fevers of unknown origin.
circulate within peripheral blood before rehoming to mucosal tissues (20, 31). This migration peaks 1 to 2 weeks after intestinal infection and may be measured by using peripheral blood mononuclear cells (PBMC) in an antibody-secreting cell (ASC) assay (19, 26) or in supernatants recovered from harvested PBMC (the “antibody in lymphocyte supernatant” [ALS] assay) (7, 31). Although ALS and ASC responses have previously been measured after immunization with oral live attenuated typhoid vaccines, detailed analyses of ALS or ASC responses in individuals with wild-type typhoid fever are lacking (21, 24). In order to gain further insight into mucosal immune responses during wild-type serovar Typhi infection, we undertook a study to characterize the serum and ALS responses to serovar Typhi among individuals with suspected typhoid fever in Bangladesh.

MATERIALS AND METHODS

Study participants and specimens.

Study participants and specimens. Individuals 3 to 59 years of age who presented to the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)-Dhaka Hospital or Dhaka Medical College Hospital with fever of 3 to 7 days duration (≥39°C), without an obvious focus of infection and lacking an alternate diagnosis, were eligible for enrollment. Because of the widespread availability of antibiotics in Bangladesh, prior use of antibiotics was not an exclusion criterion for the present study. Individuals were queried regarding headache, abdominal discomfort or pain, constipation, diarrhea, vomiting, myalgia, and loss of appetite and were assessed for lymphadenopathy, organomegaly, rash, rosy spots on the lower chest and upper abdomen, coated tongue, and relative bradycardia. We collected venous blood (5 ml from children <5 years old, and 10 ml from all others) at enrollment (day 0) and 5 and 20 days later. For children <5 years of age, 3 ml of day 0 blood was microscopically cultured; for older individuals, 5 ml of day 0 blood was microbiologically cultured. All patients suspected of having typhoid fever were initially treated with oral ciprofloxacin (10 mg/kg of body weight twice a day, up to 500 mg twice daily) or ceftaxime (4 mg/kg of body weight twice a day, up to 200 mg twice daily) or oral ciprofloxacin (10 mg/kg of body weight, twice a day, up to 500 mg twice daily) or oral ciprofloxacin (10 mg/kg of body weight, up to 500 mg twice daily), and this was transferred the supernatant to fresh tubes, centrifuged at 14,000 × g for 30 min, suspended the pellet in 10 ml of Tris buffer, and determined the protein content by the Bio-Rad protein assay (37).

Detection of typhoid-specific antibodies in specimens by ELISA. We assayed immunoglobulin A (IgA), IgG, and IgM isotype-specific antibody responses to LPS, WC, and MP by using ELISA. We coated microtiter plates (Nunc F; Nunc, Denmark) with 100 μl of LPS (2.5 μg/ml), WC (10 CFU/ml), and MP (2 μg/ml for plasma and 10 μg/ml for ALS) and blocked them with 1% bovine serum albumin in PBS (29, 31). To detect antigen-specific responses, we added 100 μl of dilutions of plasma in PBS-Tween 0.1% bovine serum albumin (1:25 for IgA and IgM and 1:50 for IgG isotypes) or 100 μl (1:2 dilution for IgA of ALS supernatant to coated plates, which were then incubated for 50 min at 37°C). After a washing step with 0.05% PBS-Tween, we detected responses using horseradish peroxidase-conjugated antibodies to either human IgA, IgG (Jackson Laborato-

RESULTS

Clinical and microbiological features in study subjects. A total of 112 febrile subjects met the inclusion criteria and completed the follow-up appointments. The median age was 24 years, with a range of 3 to 59 years (see Table 1 for the clinical characteristics). Of these, 26 participants (23%) were positive by blood culture for serovar Typhi. Of the isolated serovar Typhi organisms, 16 (62%) were resistant to ampicillin, 16 (62%) were resistant to trimethoprim-sulfamethoxazole, 24 (92%) were resistant to nalidixic acid, and 7 (27%) were resistant to ciprofloxacin, but all (100%) remained susceptible to gentamicin, cefixime, and ceftriaxone. Of the 112 study participants, 51 (46%) reported antibiotic use prior to enrollment. Of the 26 blood culture-confirmed patients, 7 (27%) reported use of ciprofloxacin prior to presenting for medical care, and all 7 grew a serovar Typhi strain that was resistant to ciprofloxacin. Another 13 individuals (12%) had a negative blood culture, but a fourfold increase of Widal titer comparing either day 5 or day 20 blood samples to day 0 samples. Of these, 5 (38%) reported ciprofloxacin use before presenting for medical care. A total of 41 participants (37%) had a negative blood culture and did not...
have a fourfold change in Widal titer but did have at least one Widal titer of $\geq 320$; of these, 18 (44%) reported use of ciprofloxacin before presenting for medical care. For the remaining 32 patients, 21 (66%) reported the use of any antibiotic before presenting for medical care. After enrollment, all patients were treated with antimicrobial agents, and 42 (38%) were hospitalized at the discretion of the attending physician. By day 5, 80 (71%) were afebrile, and by day 20 all were afebrile and well. There was no clinically evident intestinal hemorrhage or perforation, and no patient died.

**Subgrouping of study subjects for analyses.** Patients were categorized as follows. For comparison purposes, we grouped patients into a number of groups: group I, illness characterized with at least 3 days of high-grade fever and positive blood culture ($n = 26$); group II, compatible illness and a fourfold increase in Widal assay from day 0 baseline sample to convalescent period ($n = 13$); group III, compatible illness and a Widal titer of $\geq 320$ ($n = 41$); group IV, an illness suspicious for clinical typhoid fever, but with a negative blood culture and Widal titer but with an anti-serovar Typhi IgA response in the ALS assay that was $> 2$ SD above the geometric mean measured in healthy Bangladeshi controls ($> 10$ ELISA units) ($n = 14$); and group V, patients with an illness compatible with enteric fever but negative by all tests ($n = 18$). There were no patients in more than one group. Healthy controls ranged in age from 24 to 35 years (median, 27 years).

**Plasma antibody responses.** LPS-specific IgA and IgG responses were increased in patient groups I to III at all time points. FIG. 1 shows LPS-specific IgA and IgG antibody responses in plasma specimens of different study groups on different study days. Groups: group I (Gr-I), blood culture positive; GrII, fourfold change in Widal; GrIII, Widal titer of $\geq 320$; GrIV, negative culture and Widal but an anti-serovar Typhi IgA titer of $>10$ ELISA units in an ALS assay; GrV, all assays negative; HC, healthy controls. Geometric means with standard errors of the mean (SEM) are shown for day 0 (D0), day 5 (D5), and day 20 (D20). The responder rate was calculated with a cutoff of $>GM$ plus two SD of healthy controls ($55$ and $119$ ELISA units for IgA and IgG, respectively). Statistical difference compared to healthy controls: $***$, $P < 0.0001$; $**$, $P < 0.005$; $*$, $P < 0.05$. Statistical difference compared to GrV: $\phi$, $P < 0.05$.
points compared to healthy controls and compared to group V patients ($P = 0.05$ to $<0.0001$). In group IV patients, the LPS IgA response was increased at the acute stage only (Fig. 1). Compared to healthy controls, elevated LPS-IgM responses were seen at all time points and in all group I to IV patients ($P \leq 0.001$; data not shown). Patients in group V did not have increased serovar Typhi LPS-specific responses at any time point in any antibody isotype.

Anti-WC (killed WC bacterial preparation) IgA and IgG responses were seen in group I to III patients compared to healthy controls (Fig. 2). Group IV and V patients were not statistically different from healthy controls in either IgA or IgG anti-WC responses. Using a cutoff value of healthy control geometric mean plus 2 SD, 17/26 (65%) blood culture-positive patients had positive anti-MP IgA responses at the time of presentation (day 0). Of the 13 patients in group II, 8 (58%) were also positive for MP-IgA responses at presentation. Of those in group III, 18/41 (43%) patients were positive for MP-IgA responses on study day 0; 22/41 (54%) were positive on day 5, and 14/41 (44%) were positive on day 20. The MP IgG responses were only different from healthy controls in blood culture-confirmed (group I) patients at later time points and not in the day 0 plasma samples. The MP IgG responses were also not different from healthy controls in any other group at any other time point. Anti-MP IgM responses were not significantly different in any group at any time point compared to those measured in healthy controls (data not shown).

Comparison to reactivity in other illnesses using plasma. We compared the MP IgA responses in serovar Typhi blood culture-confirmed individuals for MP IgA responses in plasma obtained from patients with other confirmed causes of febrile illness or enteric disease caused by *V. cholerae* O1 (Fig. 4). The MP IgA responses were elevated at all time points in serovar Typhi blood culture-confirmed patients compared to individuals with confirmed dengue, leptospirosis, and cholera and healthy controls ($P \leq 0.05$ to $0.001$). Focusing on blood drawn at the time of presentation (day 0 sample) and using a cutoff value of geometric mean plus two SD (61 ELISA units), a positive plasma anti-MP IgA on day 0 was ca. 76% sensitive and 87% specific for blood culture-confirmed serovar Typhi infection versus all comparison groups and 76% sensitive and 100% specific when blood culture-confirmed
cases were compared to febrile cases caused by other illnesses (leptospirosis and dengue). When considering only those individuals with blood culture-confirmed serovar Typhi bacteremia (as a confirmed positive; \( n = 26 \)), and all febrile and healthy controls (as confirmed negatives; \( n = 42 \)), having a day 0 plasma MP-IgA level greater than 61 had a positive predictive value of 88%, and having a value of \( \leq 60 \) was associated with a negative predictive value of 89%.

Serovar Typhi-specific response in ALS specimens. In order to more directly evaluate mucosal IgA responses, we also measured anti-serovar Typhi responses in ALS fluid of individuals with suspected typhoid. Compared to healthy controls, the anti-WC- and anti-MP-specific IgA responses in ALS fluid in group I to IV patients was significantly elevated at all time points, including in the sample collected at the time of presentation, with the MP-specific responses being of higher magnitude than the WC-specific responses (Fig. 5).

Comparison of IgA responses in plasma and ALS specimens to MP at the acute stage of disease (day 0). We compared anti-MP IgA responses in ALS and plasma in our different groups of patients in acute-phase samples collected at the time of presentation (Table 2). Defining a positive response as being greater than two SD above the geometric mean of healthy controls (\( \geq 61 \) ELISA units, plasma; \( > 10 \) ELISA units, ALS), 21/26 (81%) of group I patients had positive plasma MP-IgA responses on day 0, and 100% of both groups had positive ALS responses. Moreover, 18/41 (43%) group III patients with a Widal titer of \( \geq 320 \) were positive in plasma, and 70% were positive in ALS. Of the remaining 32 patients, 14 (44%) had a positive ALS response, and of these 9 (28%) were also positive in plasma.

DISCUSSION

In this study, we characterized antibody responses in individuals with suspected enteric fever in Dhaka, Bangladesh. Definitive diagnosis of typhoid fever is problematic, complicating the application of any gold standard to our study. We therefore considered responses based on the key assays available in resource-poor areas of the world, blood culture positivity, and Widal serology, and grouped patients into those with definitive typhoid, an illness highly suspicious for typhoid and with a fourfold increase in Widal titer, and an illness that we characterized as possible typhoid (with a Widal titer of \( \geq 320 \)). We found serovar Typhi MP-specific IgA responses in the majority of individuals with confirmed and highly suspicious typhoid fever, suggesting that IgA responses could be used to evaluate individuals with a febrile illness compatible with typhoid.

We analyzed serovar Typhi-specific responses using three antigen preparations—LPS, WC, and a MP—and found that both IgG and IgA against LPS and WC were present across our characterized groups but that MP-specific responses were restricted to the IgA isotype (with the exception that blood cultured confirmed patients also had a positive IgG anti-MP response). These results suggest that measurement of the MP IgA responses warrants further development as a diagnostic test for individuals with suspected typhoid. The MP includes
many surface-exposed or associated serovar Typhi antigens, and whether sensitivity and specificity could be improved or decreased using a more refined preparation is currently unclear.

The presence of serovar Typhi responses in the IgA isotype may reflect the fact that *Salmonella* first enters epithelial cells of the intestinal tract, before being taken up by professional phagocytic cells, including macrophages. Although activation of systemic immune responses during *Salmonella* is well described, there are more limited data characterizing mucosal immune responses during wild-type serovar Typhi infection. Serum and salivary anti-serovar Typhi LPS IgA responses during wild-type infection have been previously reported (15), and ASC and ALS responses that measure transient presence of mucosal lymphocytes in peripheral circulation after intestinal activation have been reported in vaccinees receiving oral attenuated strains of serovar Typhi (21, 22, 24) but have not been previously described in individuals with wild-type infection. The ALS specimen that we have used to measure the responses are those derived from cells which have mucosal priming in the gut. These cells are detected in the peripheral circulation while they are rehoming back to the gut (20). The highest level of ALS response was seen during the early phase of disease, and this decreased at convalescence showing that the antigen-specific cells had decreased and hence the levels of antibodies that were being measured. For this reason, and to further characterize mucosal IgA responses during typhoid, we analyzed anti-serovar Typhi IgA responses in ALS fluid and found that anti-MP and anti-WC IgA responses were present and that anti-MP IgA responses were present in the ALS fluid in 100% of individuals with blood culture-confirmed typhoid, in 100% individuals with an illness highly suspicious for typhoid, and in 70% of individuals with possible typhoid fever in our study (a compatible illness and a Widal titer of ≥320). We were particularly intrigued by our observation that we could divide individuals with a negative blood culture and negative Widal titer into two groups: those with a positive serovar Typhi-specific IgA ALS response and those with a negative anti-serovar Typhi ALS IgA response. Whether this distinction reflects the presence or absence of active serovar Typhi infection is currently uncertain.

The ALS requires ex vivo culturing of recovered lymphocytes and, as such, may have limited clinical utility for direct development as a diagnostic assay in many resource-poor areas; however, we believe our ALS results suggest that serovar Typhi-specific IgA responses are detectable at the time of presentation for clinical care in all individuals with typhoid (confirmed cases) and that such an IgA response could be the basis of a point-of-care diagnostic assay. We also observed that a single measurement at the time of presentation of IgA in

![FIG. 4. Dot plot of MP-specific IgA responses in plasma of patients with documented serovar Typhi bacteremia (*n* = 26), confirmed dengue (*n* = 5), leptospirosis (*n* = 5), cholera (*n* = 5), and healthy controls (HC; *n* = 32). The line represents the geometric mean, and bars represent the SEM for day 0 (D0) and convalescent stages day 5 (D5) and day 20 (D20).](http://cvl.asm.org/)
plasma reacting with the serovar Typhi MP had high positive and negative predictive values in this endemic setting, including when comparing responses in individuals with other febrile illnesses difficult to clinically distinguish from typhoid fever in endemic settings (leptospirosis and dengue). Whether these results could be improved upon by use of a purified antigen or amplification of reactivity is currently unknown.

Our study has a number of shortcomings. The absence of a gold standard for diagnosing typhoid means that we can only approximate response rates across our clinical groups. The fact that many of our patients received antibiotics prior to presenting for medical care likely affected their likelihood of blood culture positivity, as well as the host-pathogen interactions that could have occurred during infection, including antibody responses. We feel, however, that our study represents an accurate clinical representation of how individuals with enteric fever present in areas of endemicity, and our blood culture positivity rate of almost 30% supports our observation that many of these individuals did indeed have serovar Typhi infection. Although serovar Typhi infection can cause a range of illness, all individuals in the present study were ill enough to seek medical care, and all had a minimum of 3 days of high level fever (with a median of 5 days). Despite these shortcomings, our results suggest that an IgA response to serovar Typhi-specific antigens is present in the peripheral circulation of many individuals with serovar Typhi infection (documented or highly suspected) at the time of presenting for clinical care and that such a response of especially the ALS specimen could be the basis of improved diagnostic point-of-care-assay for typhoid and warrants further evaluation.

**TABLE 2. MP specific IgA responses in plasma and ALS specimens at the acute stage of disease**

| Group Characteristics                  | Sample size | IgA response (%) |       |       |
|----------------------------------------|-------------|------------------|-------|-------|
|                                        |             |                  | Plasma| ALS   |
| Blood culture positive                 | 26          | 21 (81)          | 26 (100)|     |
| Fourfold Widal titer change            | 13          | 5 (38)           | 13 (100)|     |
| ≥320 Widal titer                       | 41          | 18 (43)          | 29 (70)  |     |
| Culture negative, Widal negative but a >10 ELISA unit ALS response | 14          | 4 (29)           | 14 (100)  |     |
| Culture negative, Widal negative and a ≥10 ELISA unit ALS response | 18          | 5 (28)           | 0 (0)    |     |

*Analyses of specimens on study day 1 from patients subgrouped according to different clinical and immunological characteristics. Positive responses were calculated as ≥GM plus two standard deviations of healthy control results: plasma MP-IgA response cutoff, ≥61 ELISA units; ALS MP-IgA response cutoff, >10 ELISA units.*

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