Live Oral Typhoid Vaccine Ty21a Induces Cross-Reactive Humoral Immune Responses against *Salmonella enterica* Serovar Paratyphi A and S. Paratyphi B in Humans

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Enteric fever caused by *Salmonella enterica* serovar Paratyphi A infection has emerged as an important public health problem. Recognizing that in randomized controlled field trials oral immunization with attenuated *S. enterica* serovar Typhi live vaccine Ty21a conferred significant cross-protection against S. Paratyphi B but not S. Paratyphi A disease, we undertook a clinical study to ascertain whether humoral immune responses could explain the field trial results. Ty21a immunization of adult residents of Maryland elicited predominantly IgA antibody-secreting cells (ASC) that recognize *S. Typhi* lipopolysaccharide (LPS). Cross-reactivity to S. Paratyphi A LPS was significantly lower than that to S. Paratyphi B LPS. ASC producing IgG and IgA that bind LPS from each of these *Salmonella* serovars expressed CD27 and integrin α4β7 (gut homing), with a significant proportion coexpressing CD62L (secondary lymphoid tissue homing). No significant differences were observed in serum antibody against LPS of the different serovars. Levels of IgA B memory (BM) cells to *S. Typhi* LPS were significantly higher than those against *S. Paratyphi* A or B LPS, with no differences observed between S. Paratyphi A and B. The response of IgA BM to outer membrane proteins (OMP) from *S. Typhi* was significantly stronger than that to OMP of S. Paratyphi A but similar to that to OMP of S. Paratyphi B. The percentages of IgG or IgA BM responders to LPS or OMP from these *Salmonella* strains were similar. Whereas cross-reactive humoral immune responses to *S. Paratyphi* A or B antigens are demonstrable following Ty21a immunization, they cannot explain the efficacy data gleaned from controlled field trials.

Enteric fevers are caused by the human-restricted pathogens *Salmonella enterica* serovar Typhi, *S. enterica* serovar Paratyphi A, and S. Paratyphi B (and rarely S. Paratyphi C). *S. Typhi* is estimated to cause up to 33 million cases of typhoid fever and 600,000 deaths each year worldwide (9, 10). S. Paratyphi A illness (paratyphoid A fever) is on the rise both in areas of endemicity (10, 13) and among travelers returning from regions of endemicity or epidemicity (10, 43), and the cases are increasingly caused by multidrug-resistant strains (48). Because there are no vaccines currently available to prevent enteric fevers other than typhoid fever, the development of vaccines against paratyphoid A (and perhaps paratyphoid B) fevers is a public health priority (18).

Currently, two licensed vaccines against typhoid fever (parenteral Vi polysaccharide and oral attenuated *S. Typhi* strain Ty21a) are available. Vi polysaccharide vaccine induces serum anti-Vi antibodies (33). However, because S. Paratyphi A and S. Paratyphi B do not express the Vi antigen, the Vi vaccine will not protect against these pathogens. In contrast, the licensed live oral Ty21a typhoid vaccine (Ty21a), derived from wild-type strain *S. Typhi* Ty2, has the potential to elicit cross-protection against paratyphoid fevers A and B, given the close homology in many key antigenic determinants between these *Salmonella* serovars. A few published epidemiological and retrospective studies have explored Ty21a’s possible cross-protective immunity with *S. Paratyphi* A and S. Paratyphi B infections. Results from two large-scale, randomized, placebo-controlled, double-blind (thus “gold-standard”) field trials indicate that Ty21a provides significant protection against S. Paratyphi B disease but not against S. Paratyphi A disease (3, 37, 43, 57). Two efficacy trials of Ty21a conducted in Santiago, Chile, indicated that Ty21a conferred protection from *S. Typhi* and moderate protection against paratyphoid B infections (3, 36, 37). Because few cases of paratyphoid A fever occurred during the performance of these trials, it was not possible to draw conclusions about whether Ty21a also provided partial protection against S. Paratyphi A (37). Observations from a large randomized placebo-controlled field trial in Plaju, Indonesia, strongly suggested that Ty21a is unable to confer cross-protection against S. Paratyphi A infection (57). In contrast, a retrospective study by Meltzer et al. among travelers from Israel to the Indian subcontinent suggested that Ty21a might confer some protection against *S. Paratyphi* A (42). However, in a subsequent report the authors stated that current typhoid vaccines offer no protection against S. Paratyphi A (43). Taken together, these studies strongly suggest that the currently available oral vaccine against typhoid fever either provides no protection or is not sufficiently effective against *S. Paratyphi* A.

The development of broad-spectrum live oral vaccines against the major etiologic agents causing enteric fevers will benefit from an in-depth understanding of the immunological mechanisms that are involved in the protection conferred by Ty21a against *S. Typhi* and the observed cross-protection against S. Paratyphi B. Whereas detailed studies of the immunological responses following immunization with Ty21a have been performed with *S. Typhi*, whether these immunological responses extend to *S. Paratyphi* A...
or B has not been addressed. Results from these studies suggest that the induction of specific IgA antibody-secreting cells (ASC) that recognize S. Typhi lipopolysaccharide (LPS) and the induction of memory B (B<sub>M</sub>) cells that recognize S. Typhi LPS might be among the effector mechanisms predictive of or involved in protection (15, 27, 31, 66–69, 71). It remains unknown whether these vaccine-induced humoral responses actually mediate protection or serve as a surrogate for the presence of other immune responses, such as cell-mediated immune responses (CMI), which may be more critical in mediating and conferring long-term protection. 

In this study, we evaluated the cross-reactive humoral immune responses against S. Paratyphi A and S. Paratyphi B induced following Ty21a vaccination by measuring ASC (which included the characterization of their homing potential, for which very little is known), serum antibodies (Ab), and B<sub>M</sub> cells before and after vaccination.

**MATERIALS AND METHODS**

Subjects, vaccination, and specimen collection. The study was approved by the University of Maryland Institutional Review Board. Healthy adult volunteers were recruited from the Baltimore-Washington, DC, area and University of Maryland, Baltimore, community. Prior to enrollment, the purpose of the study was explained to the subjects, and informed consent was obtained from all participants. Their medical histories were reviewed, and physical and laboratory examinations were performed to ensure that they were in good health. Any volunteer who had a history of typhoid fever or vaccination against typhoid fever was excluded from participation. Seventeen subjects (8 male and 9 female, 20 to 51 years old) were enrolled and vaccinated with four spaced doses of 2 mg S. Typhi LPS in each dose. Seventeen subjects (8 male and 9 female, 20 to 51 years old) were enrolled and vaccinated with four spaced doses of 2 mg S. Typhi LPS in each dose. Prior to enrollment, the purpose of the study was explained to the subjects, and informed consent was obtained from all participants. Their medical histories were reviewed, and physical and laboratory examinations were performed to ensure that they were in good health. Any volunteer who had a history of typhoid fever or vaccination against typhoid fever was excluded from participation.

Preparation of Salmonella lipopolysaccharide (LPS) and outer membrane protein (OMP) antigens. (i) Bacterial strains. S. Paratyphi A strain CVD 1902 and S. Typhi Ty21a have been described previously (16, 17). S. Paratyphi B strain CV 163 is an A<sub>ruA</sub> deletion mutant derived from a clinical isolate from Chile (M. M. Levine, unpublished data). Strains were grown in animal product-free LB Lennox medium (Athena ES, Baltimore, MD). Growth in liquid medium was performed overnight in flasks at 37°C and 250 rpm, followed by harvesting by centrifugation at 7,000 × g at 4°C.

(ii) LPS. S. Typhi LPS was purchased from Sigma (St. Louis, MO) (catalog no. L-7136), and S. Paratyphi A LPS and S. Paratyphi B LPS were obtained from S. Paratyphi A CVD 1902 and S. Paratyphi B CV 163, respectively, by the extraction method of Darveau and Hancock, with the addition of a final phenol purification step (11, 20). Purified LPS was resuspended in pyrogen-free water to a concentration of 20 mg/ml and stored at −70°C until used.

(iii) OMP. S. Typhi, S. Paratyphi A, and S. Paratyphi B OMGs were prepared from the strains described above by the method described by Nakaide (45), except that the final chromatography step was omitted to retain a heterogenous mixture of OMP. Final OMP preparations were dialyzed against phosphate-buffered saline (PBS) in 3.5 kDa-molecular-mass-cutoff dialysis cassettes (Thermo, Waltham, MA) and kept at 4°C for subsequent analyses. Protein concentrations were assessed by bichinchoninic acid (BCA) assay (Thermo) normalized to bovine serum albumin (BSA) standards. The relative protein composition was assessed by SDS-PAGE and Coomassie blue staining (Thermo Gelcode Blue). The identity of the expected proteins in the OMP preparations (e.g., porins) was confirmed by tryptic digestion and mass spectrometric analysis conducted at the University of Maryland Medical School Proteomics Core facility.

ASC assays. IgG and IgA antibody-secreting cells (ASC) recognizing LPS from the three Salmonella serovars were measured in circulating PBMC before and 7 days after immunization with Ty21a. A positive response was defined as an ASC count equal or greater than 8 spot-forming cells (SFC) per 10<sup>6</sup> PBMC as previously described (66, 67).

Flow cytometric determination of the expression of homing molecules and sorting of PBMC B cell subsets to measure ASC recognizing LPS. Flow cytometric measurements of the expression of homing molecules and the sorting protocol for isolating B cell subsets expressing different homing molecules were described previously (12). Briefly, freshly isolated PBMC obtained prevaccination (day 0) and 7 days postvaccination were stained with monoclonal antibodies (mAb) to CD19-phycocerythrin (PE)-Cy7 (clone J3-119; Beckman Coulter, Indianapolis, IN), CD27-PE-Cy5 (clone 1A4CD27; Beckman Coulter), CD62L-PE (L-selectin, clone Dreg-56; BD Biosciences, San Diego, CA), and integrin α<sub>4</sub>B<sub>7</sub> (clone ACT-1) conjugated to Alexa 488 using an Alexa labeling kit (Molecular Probes, Carlsbad, CA). Cells were then simultaneously sorted into 4 populations: B naïve (Bn) (CD19<sup>+</sup>CD27<sup>−</sup>) or B memory (B<sub>M</sub>) (CD19<sup>+</sup>CD27<sup>+</sup>) expressing CD62L but not integrin α4β7 (B<sub>M</sub> lymph node [LN]) (CD62L<sup>+</sup> integrin α4β7<sup>−</sup>), B<sub>M</sub> expressing integrin α4β7 but not CD62L (B<sub>M</sub>α4β7<sup>−</sup>CD62L<sup>+</sup> integrin α4β7<sup>−</sup>), or B<sub>M</sub> expressing both integrin α4β7 and CD62L (B<sub>M</sub>α4β7<sup>−</sup>CD62L<sup>+</sup> integrin α4β7<sup>−</sup>). Four-way sorting was performed in a MoFlo flow cytometer/cell sorter system (Beckman-Coulter). Puriﬁed populations were 86% to 96% (the gating strategy is shown in Fig. S1 in the supplemental material). IgG and IgA ASC recognizing S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS in each sorted population were measured as described above.

**Serum Ab assays.** IgG and IgA serum antibodies (Ab) to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS were measured by enzyme-linked immunosorbent assay (ELISA) (71). Endpoint titers were calculated through linear regression as the inverse of the serum dilution that produces an optical density (OD) of 0.2 above the value for the blank. Postvaccination fold increases of anti-LPS Ab titers were calculated as titers postvaccination divided by the corresponding prevaccination titers × 100. Seroconversion was defined as a ≥4-fold increase in postvaccination Ab titer at any time point (day 7 or 42) postvaccination compared to prevaccination.

IgA B<sub>M</sub> ELISpot assay. The method used for the enzyme-linked immunosorbent spot (ELISpot) assay has been described previously (7). Briefly, frozen PBMC were thawed and expanded with B cell expansion medium consisting of 5 μM β-mercaptoethanol (β-ME) (Bio-Rad, Hercules, CA), 1:100,000 pokeweed mitogen (kindly provided by S. Crotty), 6 μg/ml CpG-2006 (Sigma), and 1:10,000 Staphylococcus aureus Cowan (Sigma) in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (CellGiro, Manassas, VA), 50 μg/ml gentamicin (HyClone, Logan, UT), 2 mM l-glutamine, 2.5 mM sodium pyruvate, 10 mM HEPES, and 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD) (complete RPMI). Cells were expanded for 5 days (1.5 × 10<sup>6</sup> cells/well in 6-well plates). Supernatants were collected for antibody-in-lymphocyte-supernatant (ALS) measurements, and expanded PBMC were used immediately in B<sub>M</sub> ELISpot assays by seeding them on nitrocellulose plates (Mahan; Millipore, Billerica, MA) and incubated with anti-human IgA (Jackson ImmunoResearch Lab, West Grove, PA) (5 μg/ml) diluted in PBS as described previously (71).

Adequate expansion of B<sub>M</sub> cells was assessed by the frequency of total IgA detected by ELISpot assay as described previously (71). Any specimen that had fewer spot-forming cells (SFC) than the 5th percentile of the total IgA SFC per 10<sup>6</sup> expanded PBMC (3,965/10<sup>6</sup> cells) was excluded from analysis. One volunteer was excluded from the analysis based on this criterion. As described, a cutoff for postvaccination responders was de-
IgG S.

ASC responses that recognize S. Typhi, S. Paratyphi A (Para A), and S. Paratyphi B (Para B) in Ty21a vaccinees (n = 16) before and 7 days after immunization are shown. Data are expressed as mean ± standard error of the mean (SEM). #, P < 0.05 compared to the respective day 0 value. **, P < 0.001; *, P < 0.05 (Wilcoxon matched-pair test).

Measurement of ALS of BM cultures. In the culture supernatants collected after 5 days of expansion for BM assay (see “IgA BM ELISpot assay” above), IgG and IgA antibodies against S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS and OMP were measured by ELISA as described above. Positive responders by ALS were defined as those subjects who exhibited an increase of at least 2-fold in the specific Ab titers at any of the postvaccination times (day 42, 84, or 180) compared to their corresponding pre-vaccination levels (day 0).

Statistical analysis. Statistical comparisons were carried out using nonparametric Wilcoxon matched-pair or Mann-Whitney U tests as indicated. Correlations between two parameters were examined by Spearman’s correlation. Differences with P values of <0.05 (two tailed) were considered significant. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla CA).

RESULTS

Induction in Ty21a vaccinees of ASC that recognize S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS. The appearance of antigen-specific ASC within the first week after immunization with attenuated oral vaccines, including Ty21a, is often used as a parameter to evaluate immunogenicity. To determine whether there is cross-reactivity to LPS purified from S. Typhi, S. Paratyphi A, and S. Paratyphi B, we measured the presence of specific IgA and IgG ASC before immunization and on day 7 postvaccination using PBMC immediately after isolation from Ty21a vaccinees. Out of 17 volunteers recruited, PBMC from one volunteer were not available on day 7 for ASC measurements. As shown in Fig. 1A, IgA ASC responses that recognize S. Typhi LPS (median, 47/10⁶ PBMC) were detected in 13 out of 16 (81%) volunteers in their day 7 postvaccination specimens compared to the corresponding pre-vaccination levels. Similarly, 12 out of 16 volunteers (75%) exhibited IgA ASC responses that recognized S. Paratyphi A and S. Paratyphi B LPS (median, 27 and 45.5/10⁶ PBMC, respectively). However, the magnitude (mean ± standard error [SE]) of the LPS IgA ASC responses directed to S. Typhi was significantly higher than that to S. Paratyphi A or S. Paratyphi B LPS, and the magnitude of the responses to S. Paratyphi B was significantly higher than that to S. Paratyphi A (Fig. 1A and Table 1).

We also measured the IgG ASC responses to the different LPS antigens in these specimens (Fig. 1B). The percentage of positive responders for IgG ASC that recognize S. Typhi LPS (median, 22/10⁶ PBMC), observed in 11 out of 16 (69%) was higher, albeit not statistically significantly, than that of positive responders for IgG ASC recognizing S. Paratyphi A LPS (median, 6/10⁶ PBMC), observed in 7 out of 16 (44%), or S. Paratyphi B LPS (median, 11.5/10⁶ PBMC), observed in 8 out of 16 (50%). However, the magnitude of IgG ASC responses to S. Typhi LPS was significantly higher than that to S. Paratyphi A LPS but not significantly different from that to S. Paratyphi B LPS (Fig. 1B and Table 1). The IgG ASC responses to S. Paratyphi B, albeit somewhat higher than those to S. Paratyphi A, did not reach statistical significance (Table 1). IgA ASC responses predominated over the corresponding IgG ASC responses to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS (Table 1).

Characterization of LPS-specific ASC homing patterns. Since the gut microenvironment is the first line of defense against enteric diseases, we investigated the homing characteristics of the ASC that recognize LPS induced by immunization with Ty21a. To

### TABLE 1 LPS ASC responses to S. Typhi, S. Paratyphi A, and S. Paratyphi B

<table>
<thead>
<tr>
<th>Ig</th>
<th>Serovar</th>
<th>ASC/10⁶ PBMC (mean ± SE)*</th>
<th>P value compared to:</th>
<th>Hierarchy of response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. Typhi</td>
<td>81.13 ± 19.97*</td>
<td>S. Typhi</td>
<td>S. Typhi &gt; S. Paratyphi B &gt; S. Paratyphi A</td>
</tr>
<tr>
<td></td>
<td>S. Paratyphi A</td>
<td>42.94 ± 12.49*</td>
<td>0.005</td>
<td></td>
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<tr>
<td></td>
<td>S. Paratyphi B</td>
<td>61.06 ± 16.97*</td>
<td>0.038</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>S. Typhi</td>
<td>28.13 ± 6.61</td>
<td>S. Typhi = S. Paratyphi B &gt; S. Paratyphi A</td>
<td></td>
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<tr>
<td></td>
<td>S. Paratyphi A</td>
<td>15.56 ± 5.22</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. Paratyphi B</td>
<td>29.13 ± 12.18</td>
<td>0.5</td>
<td></td>
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</table>

* *, P < 0.05 compared to corresponding serovar IgG ASC value.
study the homing potential of circulating ASC, we sorted fresh PBMC obtained 7 days after immunization with Ty21a into four distinct subpopulations of CD19<sup>+</sup> B cells. The gating and purity of the sorted populations are shown in Fig. S1 in the supplemental material. Sorted B cell subsets were immediately plated in S. Typhi LPS-coated plates as described in Materials and Methods. ASC frequencies for each of the subpopulations revealed that virtually all IgA and IgG ASC that recognize LPS were observed in the BM (CD19<sup>+</sup>CD27<sup>+</sup>) subset (Fig. 2A and D). The majority of IgA and IgG ASC recognizing LPS were observed among BM cells that expressed the gut homing molecule integrin α4β7 in the absence of CD62L (BM gut; i.e., endowed with the potential to home to the gut). However, it is important to note that significant proportions of both IgG and IgA ASC recognizing LPS were also observed among CD19<sup>+</sup>CD27<sup>-</sup>CD62L<sup>+</sup>α4β7<sup>+</sup> cells, which appear to have the capacity to home to both the gut and peripheral lymphoid tissues (BM<sub>LN</sub>/gut). Virtually no ASC were observed in plates seeded with Bn cells (CD19<sup>+</sup>CD27<sup>-</sup>) or BM<sub>LN</sub> cells that expressed the lymph node homing receptor CD62L in the absence of integrin α4β7 (BM<sub>LN</sub>) (Fig. 2A and D).

We next explored the possibility that differences in the homing characteristics of ASC elicited following immunization with Ty21a that are reactive to S. Paratyphi A and S. Paratyphi B LPS preparations could help explain the observed moderate cross-protection to infections with S. Paratyphi B, but not to S. Paratyphi A, reported for Ty21a vaccinees. As can be seen in Fig. 2, no differences were observed in the homing characteristics of the various IgA and IgG ASC B cell subsets recognizing LPS from S. Typhi (Fig. 2A and D), S. Paratyphi A (Fig. 2B and E), or S. Paratyphi B (Fig. 2C and F).

**Induction of serum Ab to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS in Ty21a vaccinees.** We next evaluated whether there were differences in the serum antibody levels to LPS from S. Typhi, S. Paratyphi A, and S. Paratyphi B following Ty21a vaccination. Sera were collected before (day 0) and 7 and 42 days following immunization. The mean serum Ab titers against LPS from S. Typhi, S. Paratyphi A, and S. Paratyphi B were significantly elevated on days 7 and 42 postvaccination compared to their prevaccination levels, with the highest levels observed in day 7 samples (Fig. 3). Postvaccination fold increases of anti-LPS IgA Ab titers declined markedly from day 7 to day 42 (Fig. 3A). No significant differences were observed in the seroconversion rates for IgA Ab titers against S. Typhi (8 out of 17; 47%), S. Paratyphi A (6 out of 17; 35%), and S. Paratyphi B (6 out of 17; 35%) LPS.

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**FIG 2** Homing characteristics of ASC in Ty21a vaccinees. Shown are IgA (A, B, and C) and IgG (D, E, and F) ASC specific for LPS from S. Typhi (A and D), S. Paratyphi A (B and E), and S. Paratyphi B (C and F) in sorted subsets from 4 individual Ty21a vaccinees. Data for each sorted subset as defined in the legend box are shown as the percentages of total LPS-specific ASC observed in the corresponding subject (ASC in each sorted subset/total ASC detected in the corresponding subject × 100). Error bars indicate SEMs. ***, P < 0.01; ****, P < 0.001. #, P < 0.01 compared to the corresponding BM<sub>LN</sub> subset (Mann-Whitney test, 2 tailed).
Similarly, no differences were observed in the mean ± SE of the peak postvaccination (from either day 7 or 42) IgA Ab fold increases against S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS antigens (Table 2).

Serum IgG anti-LPS Ab titer measurements were also performed against all three LPS preparations. Similar to the results observed with IgA, significantly elevated IgG anti-LPS levels were observed on days 7 and 42 postvaccination compared to the corresponding prevaccination levels. However, as shown in Fig. 3B, in contrast to the observations with IgA (Fig. 3A), the postvaccination fold increases of anti-LPS IgA Ab titers did not decline markedly from day 7 to day 42. The seroconversion rates for IgG anti-LPS Ab titers against S. Typhi (9 out of 17; 53%), S. Paratyphi A (6 out of 17; 35%), and S. Paratyphi B (5 out of 17; 29%) were not significantly different. No differences were observed in the mean ± SE of the peak postvaccination IgG LPS Ab fold increases for S. Typhi, S. Paratyphi A, and S. Paratyphi B (Table 2).

Taken together, these anti-LPS serological results indicate the lack of statistically significant differences when considering either fold increases, seroconversion rates, or mean titers (data not shown) in IgA or IgG to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS (Table 2).

Induction of IgA B<sub>M</sub> cells to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS and OMP, measured by ELISpot assay, in Ty21a vaccinees. The induction of antigen-specific B<sub>M</sub> cells, which can be measured in cryopreserved PBMC, is now considered to be a widely accepted immunological tool to study the long-term humoral immunity elicited following vaccination or natural infection (7, 26, 58, 59, 71). Therefore, it was of importance to determine whether Ty21a immunization elicits IgA B<sub>M</sub> anti-LPS and anti-OMP responses that are cross-reactive among LPS and OMP preparations from S. Typhi, S. Paratyphi A, and S. Paratyphi B. Study specimens were collected before (day 0) and after (days 42, 84, and 180) immunization to determine the persistence of B<sub>M</sub> responses. As shown in Fig. 4A, LPS-specific IgA B<sub>M</sub> responses were induced against all 3 LPS preparations. The mean peak frequencies of IgA LPS-specific B<sub>M</sub> responses for S. Typhi and S. Paratyphi B were significantly elevated from their corresponding prevaccination levels, while the postvaccination rises for S. Paratyphi A showed a strong trend but did not reach statistical significance (Fig. 4A). The dominant postvaccination responses were directed toward S. Typhi LPS and were significantly higher than those observed against both S. Paratyphi A and B (Fig. 4A). No significant differences were observed between peak responses to S. Paratyphi A and S. Paratyphi B (P = 0.15). Similarly, when the net postvaccination increases were calculated by subtracting the prevaccination level in each volunteer from the respective postvaccination peak frequencies, significantly higher postvaccination levels were observed toward S. Typhi LPS than toward S. Paratyphi A or S. Paratyphi B (Fig. 4B and Table 3). Net postvaccination increases to S. Paratyphi B were not significantly higher than those directed to S. Paratyphi A (Fig. 4B and Table 3). The percentage of IgA B<sub>M</sub> cell-positive responders for S. Typhi (9 out of 16; 56%) showed a trend but was not significantly different from those for S. Paratyphi A (5 out of 16; 31%) or S. Paratyphi B (6 out of 16; 38%).

![FIG 3 LPS-specific serum antibody responses in Ty21a vaccinees. Shown are postvaccination fold increases in serum anti-LPS antibodies to S. Typhi-, S. Paratyphi A (Para A)-, and S. Paratyphi B (Para B)-specific IgA (A) and IgG (Panel B) in Ty21a vaccinees (n = 17). The dashed horizontal lines represent 4-fold increases, the cutoff for seroconversion. Error bars indicate SEMs. **, P < 0.01; *, P < 0.05 (by Wilcoxon matched-pair test, two tailed).](http://cvi.asm.org/)

**TABLE 2 Serum LPS antibody responses to S. Typhi, S. Paratyphi A, and S. Paratyphi B**

<table>
<thead>
<tr>
<th>Ig</th>
<th>Serovar</th>
<th>Peak fold Ab increase&lt;sup&gt;a&lt;/sup&gt; (mean ± SE)</th>
<th>P value compared to:</th>
<th>Hierarchy of response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. Typhi</td>
<td>S. Paratyphi A</td>
</tr>
<tr>
<td>IgA</td>
<td>S. Typhi</td>
<td>5.42 ± 1.54</td>
<td>0.89</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>S. Paratyphi A</td>
<td>3.50 ± 0.40</td>
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<tr>
<td></td>
<td>S. Paratyphi B</td>
<td>4.47 ± 0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>S. Typhi</td>
<td>4.49 ± 0.76</td>
<td>0.56</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>S. Paratyphi A</td>
<td>4.46 ± 0.99</td>
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<tr>
<td></td>
<td>S. Paratyphi B</td>
<td>3.47 ± 0.53</td>
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</table>

<sup>a</sup> Peak postvaccination fold increase in Ab titer at day 7 or 42 compared to corresponding prevaccination level.
Outer membrane proteins (OMPs), which include porins of Gram-negative bacteria, are highly immunogenic. Therefore, IgA B\(_M\) responses specific to the OMP preparations described in Materials Methods were also measured. As shown in Fig. 5A, OMP-specific IgA B\(_M\) responses were elicited against all three OMP preparations, as evidenced by significant or very strong trends in increased responses in postvaccination peak levels compared to the corresponding prevaccination levels. Interestingly, unlike observations with LPS, the peak responses directed to \(S.\) Typhi OMP were similar in magnitude to those against \(S.\) Paratyphi B OMP (Fig. 5A). In contrast, IgA B\(_M\)-specific OMPs from both \(S.\) Typhi and \(S.\) Paratyphi B were significantly higher than those against \(S.\) Paratyphi A (Fig. 5A).

Similar results were observed when analyzing net increases in postvaccination peak B\(_M\) responses specific for \(S.\) Typhi and \(S.\) Paratyphi A (Fig. 5B and Table 3). Net increases to \(S.\) Paratyphi A were significantly lower than those to \(S.\) Typhi, while only a trend to exhibit lower net increases was observed when comparing responses to \(S.\) Paratyphi A and \(S.\) Paratyphi B (Fig. 5B and Table 3). The percentage of IgA B\(_M\)-positive responders to OMP for \(S.\) Typhi (13 out of 16; 81\%) was not significantly different from that to \(S.\) Paratyphi A (10 out of 16; 63\%) or \(S.\) Paratyphi B (11 out of 16; 69\%).

### TABLE 3 IgA B\(_M\) responses to \(S.\) Typhi, \(S.\) Paratyphi A, and \(S.\) Paratyphi B

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Serovar</th>
<th>Peak % increase(^a) (mean ± SE)</th>
<th>(P) value compared to:</th>
<th>Hierarchy of response</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>(S.) Typhi</td>
<td>0.15 ± 0.06</td>
<td></td>
<td>(S.) Typhi &gt; (S.) Paratyphi A = (S.) Paratyphi B</td>
</tr>
<tr>
<td></td>
<td>(S.) Paratyphi A</td>
<td>0.053 ± 0.02</td>
<td>0.002</td>
<td>(S.) Paratyphi A = (S.) Paratyphi B</td>
</tr>
<tr>
<td></td>
<td>(S.) Paratyphi B</td>
<td>0.064 ± 0.019</td>
<td>0.038</td>
<td>(S.) Paratyphi B ≥ (S.) Paratyphi A</td>
</tr>
<tr>
<td>OMP</td>
<td>(S.) Typhi</td>
<td>0.234 ± 0.047</td>
<td></td>
<td>(S.) Typhi ≥ (S.) Paratyphi B ≥ (S.) Paratyphi A</td>
</tr>
<tr>
<td></td>
<td>(S.) Paratyphi A</td>
<td>0.135 ± 0.046</td>
<td>0.039</td>
<td>(S.) Typhi ≥ (S.) Paratyphi B ≥ (S.) Paratyphi A</td>
</tr>
<tr>
<td></td>
<td>(S.) Paratyphi B</td>
<td>0.201 ± 0.054</td>
<td>0.41</td>
<td>(S.) Typhi ≥ (S.) Paratyphi B ≥ (S.) Paratyphi A</td>
</tr>
</tbody>
</table>

\(a\) Peak postvaccination increase in percentage of specific B\(_M\)/total IgA SFC at day 42, 84, or 118 minus the corresponding prevaccination level.
Taken together, these results suggest that the IgA B_M responses to LPS and OMP follow the general trend of the strongest responses being observed against S. Typhi, followed by S. Paratyphi B and then S. Paratyphi A.

**Induction of IgA and IgG B_M cells to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS and OMP, determined by ALS assay, in Ty21a vaccinees.** The measurement of antigen-specific Ab in supernatants of expanded cultures can be used as a proxy for the presence of B_M cells, especially in situations where there are limitations in cell availability. This has been validated in a few studies, including our own, which reported positive correlations of LPS responses between antibody-in-culture-supernatant (ALS) and B_M ELISpot assays (26, 71). In the present studies we observed similar correlations between B_M and ALS assays with OMP preparations (see Fig. S2 in the supplemental material). Because of cell limitations, we were unable to measure IgG antigen-specific B_M by ELISpot assay in this study. However, sufficient culture supernatants were available from 15 of 16 subjects to enable the measurement of IgG and IgA to LPS by ALS assay. No significant differences were observed in the percentages of IgA responders to S. Typhi, S. Paratyphi A, and S. Paratyphi B LPS (Fig. 6A). Except in a few volunteers, the levels of IgG anti-LPS were below the detection level. Only 20 to 25% of the subjects were found to be IgG responders to the LPS preparations by ALS assay (Fig. 6A). In contrast, both IgA and IgG Ab to all three OMP preparations were observed in most volunteers by ALS assays. However, no significant differences in the percentage of IgA and IgG responders to any of the three strain-specific OMP preparations were observed (Fig. 6B).

**DISCUSSION**

Results from prospective, randomized, placebo-controlled large-scale field trials demonstrate that Ty21a oral typhoid vaccine can protect against S. Paratyphi B (3, 37) but not against S. Paratyphi A (57). We undertook to determine whether cross-reacting immune responses to S. Paratyphi B and A can explain the observed differences, beginning with a detailed analysis of humoral B cell responses.

Vaccines typically protect from disease and/or infection by eliciting effector immunity and immunological memory (38) and have the potential to elicit cross-protection to related organisms if they express similar protective antigens. For example, these serovars share some O antigenic determinants, e.g., O antigen (37, 40), and some of the proteins expressed in these Salmonella species, e.g., OmpC and OmpF, share a considerably degree of homology (46).

Three doses of Ty21a in enteric-coated capsules have been shown to confer 62% protective efficacy against typhoid fever over a period of 7 years of follow-up (35). The identification of the protective antigens and immune mechanisms responsible for protection following Ty21a vaccination is severely limited by the fact that S. Typhi is a human-restricted pathogen, which limits these studies to humans. In spite of these restrictions, extensive studies on the immunological responses elicited by typhoid vaccines were carried out in subjects immunized with Ty21a, as well as other novel vaccine candidates (31, 39, 41, 50–54, 61–63, 66–69, 71–73). Although these studies have advanced our knowledge on the humoral and cell-mediated immune responses elicited by this vaccine, the "true" effector immunological mechanisms responsible for protection remain elusive. Nevertheless, the appearance of LPS-specific ASC in circulation and of serum Ab following vaccination with Ty21a has been proposed as a surrogate of protection based on the observation that their magnitudes increase with the number of doses administered (27), a fact that tracks the protective efficacy with increasing doses of Ty21a in field trials (14). Thus, the present study was directed to uncover the immunological mechanisms which could explain the observed cross-protection in Ty21a vaccinees against S. Paratyphi B, but not S. Paratyphi A, by focusing on adaptive humoral immune responses to these 3 Salmonella serovars. These studies might also provide important information to accelerate the development of S. Paratyphi A vaccines.

Our results on the induction of IgA and IgG LPS ASC following immunization with Ty21a showed that the magnitude of these responses was significantly higher to S. Typhi than to either S. Paratyphi A or S. Paratyphi B. Interestingly, the IgA LPS ASC responses to S. Paratyphi B were significantly higher than those to S. Paratyphi A, but only a similar trend was observed in IgG LPS ASC. It is possible that the latter trend in LPS IgG ASC was the result of the relatively limited number of subjects evaluated. Future studies with increased numbers of volunteers should establish the validity of this argument. The observed induction of predominantly mucosal IgA ASC is similar to that previously reported with Ty21a and other attenuated candidate typhoid vaccines (28, 31, 66–68, 71). However, to our knowledge this study is the first to demonstrate cross-reactive IgA ASC responses to S. Paratyphi A and S. Paratyphi B LPS following Ty21a vaccination.

Serum antibody responses to LPS and other S. Typhi antigens also traditionally have been measured in the study of immunogenicity of Ty21a and other candidate typhoid vaccines (15, 31, 34, 61, 64, 66, 70, 71). Although in a few reports serum Ab to S. Typhi have been demonstrated to kill Salmonella ex vivo (21, 39, 69), this is unlikely to represent the ultimate operative mechanism of protection. In the present study, the kinetics of Ab responses to LPS were similar to our previous observations with S. Typhi candidate vaccine CVD 909 (71); however, no differences were observed in the kinetics of induction of serum IgG or IgA antibodies to LPS from S. Typhi, S. Paratyphi A, and S. Paratyphi B (in either magnitude, percentage of responders, fold increases, or persistence).

The cross-reactive humoral responses to LPS are likely directed toward shared O antigen 12, the triscarhide (mannose-rhamnose-galactose) repeating unit that comprises the backbone common to Salmonella groups A, B, and D. A hexose linked to the mannoside residue comprises the immunodominant epitope that...
results in serogroup specificity. This hexose is a paratose in group A, an abequose in group B, and a tyvelose in group D. However, the trisaccharide repeat backbone (O antigen 12) is identical among the three O serogroups (37, 40). Ty21a is mutated in the gene for the UDP-galactose-4-epimerase; consequently, Ty21a cannot de novo synthesize smooth O polysaccharide (OPS). However, if provided exogenous galactose, it can make smooth LPS. Thus, Ty21a is grown in fermentors in broth containing 0.01% galactose, which is common to all responses may also be directed against epitopes of the core polysaccharide, which is common to all Salmonella serovars. However, the lack of cross-protection against S. Paratyphi A in the field trials argues against the possibility that these cross-reactive shared epitopes play a critical role in cross-protection. Of note, Ab against the trisaccharide backbone also demonstrate lower protection in animal models than those directed to the serogroup OPS-specific epitopes (6). Taken together, these results support the contention that the minor differences observed between ASC and antibody responses to S. Paratyphi A and S. Paratyphi B LPS are unlikely to provide an immunological basis for the lack of cross-protection from S. Paratyphi A infection observed in the field trials.

We also explored whether the differences in the observed cross-protection to S. Paratyphi B, but not S. Paratyphi A, might be found in differences in the homing potentials of anti-LPS B M cell subsets elicited by Ty21a immunization. It has been shown that following oral Ty21a vaccination, circulating ASC in peripheral blood expressed the integrin α4β7 gut homing receptor (29, 47). However, largely due to technical limitations, our knowledge of the relative proportions of ASC homing to the gut and to peripheral lymphoid tissues is rather limited. The CD27 molecule is present in B cells that have undergone the process of hypermutation after encountering antigens, and therefore it is expressed in ASC and B M cells (1, 32). Regarding homing, CD62L (L-selectin) is required by leukocytes to enter secondary lymphoid tissues via high endothelial venules, while integrin α4β7 is a key molecule involved in gut homing (2, 4, 36). Thus, ASC populations (CD19+CD27+) which express integrin α4β7, but not CD62L, are effector B cells that are destined to migrate exclusively to the gut mucosa, whereas cells expressing CD62L but not integrin α4β7 are destined to home to secondary lymphoid tissues. Although the homing potential and the activity of ASC and B M cells that express both integrin α4β7 and CD62L population are not very well understood, previous studies have suggested that they have the potential to migrate to both gut and peripheral lymph nodes (4, 25).

To uncover the phenotypic and homing characteristics of S. Typhi LPS-specific ASC elicited by Ty21a immunization, we developed a novel approach to study the proportions of specific ASC which exhibit the characteristics of B naive or ASC/B M cells and that have the potential to home to secondary lymphoid tissues and/or gut by flow cytometric cell sorting. The results clearly indicate that the vast majority of both IgA and IgG anti-S. Typhi LPS-specific ASC express the gut homing molecule integrin α4β7 in the absence of CD62L. Importantly, we observed for the first time that a significant proportion of these specific ASC are also endowed with the capacity to home to both the gut and peripheral secondary lymphoid tissues, including peripheral lymphoid tissues. Only a very small minority of LPS-specific ASC express exclusively CD62L. The fact that we observed identical homing patterns in ASC cross-reactive to S. Paratyphi A and S. Paratyphi B strongly suggests that differences in the homing patterns of IgG and IgA anti-LPS ASC are unlikely to explain the differences in cross-protection to S. Paratyphi B and S. Paratyphi A observed in epidemiological studies.

Vaccines protect from infection or disease by eliciting effector immune responses as well as by the generation of T and B memory cells, which are primarily responsible for the longevity of the response (55). The recent development of a technique to measure B M cells in PBMC has been used to further evaluate and understand the protective immune memory induced by vaccines or natural infections (8, 19, 26, 58, 59, 71). We have previously demonstrated that Ty21a vaccination is capable of eliciting IgA B M responses to the T-independent antigen LPS and both IgA and IgG B M responses to T-dependent protein antigens (71).

To evaluate whether differences in the induction of B M cells to S. Typhi, S. Paratyphi A, and S. Paratyphi B could be responsible for the observed cross-protection between S. Typhi and S. Paratyphi B, we measured the induction of B M responses to both T-independent (i.e., LPS) and T-dependent (e.g., outer membrane protein [OMP]) antigens from these Salmonella serovars in Ty21a vaccines. OMPs were selected for several reasons. OMPs have been demonstrated to elicit persistent antibody responses against S. Typhi in mice (22–24). Moreover, rises in serum antigen antibody titers have been observed in healthy volunteers vaccinated with porins and following typhoid fever infections (5). Based on these findings, these proteins have been proposed as candidate vaccines against typhoid fever (49).

Previous studies have shown that immunity elicited by oral vaccination or infection elicits predominantly IgA responses (19, 58, 60). Thus, due to limitations in specimen availability, we performed B cell ELISpot assays to measure IgA B M only. However, the IgG B M responses were measured using ALS assays, which have shown to be a reliable alternative for measuring B M responses (26, 30, 71). Of note, in the current study we observed very strong correlations between the data for IgA to OMP determined by ALS and the B M data determined by ELISpot, confirming the concordance between these 2 B M cell assays and the validity of the data obtained by using these two different methods.

We observed that Ty21a induced predominantly IgA B M responses against S. Typhi LPS and OMP. However, in spite of the fact that cross-reactivity against both S. Paratyphi A and S. Paratyphi B LPS was apparent, no significant differences were observed in these B M responses. In contrast, OMP-specific B M responses elicited by immunization with Ty21a were similar against both S. Typhi and S. Paratyphi B but lower against S. Paratyphi A. However, no significant differences in the proportion of responders were observed against LPS from the 3 Salmonella serovars. Of note, IgG B M responses against LPS antigens from all three strains were detectable in just a few individuals. This low proportion of IgG B M responders for T-I antigens (e.g., LPS and Vi polysaccharide) is similar to that previously observed with oral vaccination with Ty21a or CVD 909 but not with T-D antigens such as flagella (71). It could be argued that T-I antigens are less potent in inducing long-term IgG B M responses and that the oral route preferentially induces mucosal immune responses.

In sum, the evidence presented in this report supports the notion that humoral responses to key antigens (LPS and OMP) do not play the dominant role in the cross-protective immunity observed between S. Typhi and S. Paratyphi B, but not S. Paratyphi
A, following Ty21a immunization. However, we cannot rule out that other related humoral responses not measured in this study, such as antibody responses to other Salmonella serovar common antigens (e.g., flagella or core OPS), or the functional ability of antibodies (e.g., avidity, opsonophagocytic, and serum bactericidal activity) plays a role in cross-protection. We are currently performing assays on the functional antibody immune responses induced by Ty21a against S. Paratyphi A and S. Paratyphi B to investigate whether they could explain the lack of correlation of the measured humoral responses and protection in field studies.

An important aspect of the pathogenesis of enteric fevers is S. Typhi’s ability to survive and replicate intracellularly in its dissemination and persistence in the host (44, 74). Thus, it is likely that CM1 plays a key role, maybe the dominant role, in protection from S. Typhi infection. In fact, a growing body of literature shows that oral immunization of subjects with Ty21a and other attenuated S. Typhi vaccine candidates elicits a wide array of specific effector and memory T cell responses, including, among others, proliferative responses, proinflammatory cytokines, and cytotoxic T lymphocytes restricted by classical and HLA-E major histocompatibility complex (MHC) molecules (31, 41, 49–54, 61–63, 65–68, 72, 73, 75), that might underlie the long-term efficacy of the Ty21a vaccine. Further studies directed to evaluate whether differences in CM1 to S. Typhi, S. Paratyphi A, and S. Paratyphi B antigenic stimulation are observed following Ty21a immunization might provide a mechanistic basis for the observed cross-protection between S. Typhi and S. Paratyphi B elicited by Ty21a. This has the potential to dramatically accelerate the development of broad-spectrum live oral vaccines against the major etiologic agents causing enteric fever. One could envision the development of a bivalent vaccine consisting of an attenuated strain to prevent S. Typhi and S. Paratyphi B disease and another to prevent S. Paratyphi A disease. Such a vaccine would be much simpler to develop, manufacture, and ensure consistency for than a trivalent vaccine.

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REFERENCES


