Antibody Avidity in Humoral Immune Responses in Bangladeshi Children and Adults following Administration of an Oral Killed Cholera Vaccine

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Antibody avidity for antigens following disease or vaccination increases with affinity maturation and somatic hypermutation. In this study, we followed children and adults in Bangladesh for 1 year following oral cholera vaccination and measured the avidity of antibodies to the T cell-dependent antigen cholera toxin B subunit (CTB) and the T cell-independent antigen lipopolysaccharide (LPS) in comparison with responses in other immunological measurements. Children produced CTB-specific IgG and IgA antibodies of high avidity following vaccination, which persisted for several months; the magnitudes of responses were comparable to those seen in adult vaccinees. The avidity of LPS-specific IgG and IgA antibodies in vaccinees increased significantly shortly after the second dose of vaccine but waned rapidly to baseline levels thereafter. CTB-specific memory B cells were present for only a short time following vaccination, and we did not find significant memory B cell responses to LPS in any age group. For older children, there was a significant correlation between CTB-specific memory T cell responses after the second dose of vaccine and CTB-specific IgG antibody avidity indices over the subsequent year. These findings suggest that vaccination induces a longer-lasting increase in the avidity of antibodies to a T cell-dependent antigen than is measured by a memory B cell response to that antigen and that early memory T cell responses correlate well with the subsequent development of higher-avidity antibodies.

Cholera is an acute dehydrating diarrheal disease caused by toxigenic strains of *Vibrio cholerae* serogroups O1 and O139 (1, 2). Cholera is endemic in over 50 countries globally; in these countries, young children bear a large burden of disease (3–5). Cholera also causes significant morbidity and death through epidemics and outbreaks in countries in which the disease is not endemic. Along with efforts to improve access to clean water and sanitation, the WHO has advocated the use of oral cholera vaccines in areas with both epidemic and endemic disease (6). Unfortunately, young children receiving oral killed cholera vaccine achieve lower protective efficacy and a shorter duration of protection than older children and adults (7), the reasons for which are unknown.

The mechanism of protection against cholera infection is still not fully understood. In studies of household contacts of patients with cholera, we demonstrated previously that levels of IgA antibodies as well as memory B cell responses to lipopolysaccharide (LPS), a T cell-independent antigen, on exposure in the household are associated with protection against disease (8, 9). We have also demonstrated that adults with cholera have significant elevations in circulating cholera toxin-specific memory B cells that persist for at least 360 days after illness (10). Adult vaccinees also have cholera toxin-specific memory B cells for up to 180 days after vaccination but do not develop significant memory B cell responses to LPS (11).

Antibody production after infection or vaccination involves the process of affinity maturation and somatic hypermutation of antigen-specific B cells, most effectively in response to antigen-specific follicular helper T cells (T<sub>FH</sub>) in germinal centers (GCs). Antibody avidity has been used as a measure of functional maturation of the humoral immune response, and increases in antibody avidity over time have been shown after both infection and vaccination (12–14). We showed previously, in adults, that the avidity of antibodies against both cholera toxin B subunit (CTB) and LPS increases following cholera infection or cholera vaccination and correlates with the levels of memory B cells against the respective antigens; the durability of high-avidity antibodies is longer in patients than in vaccinees (15).

The reasons for the poor efficacy of oral cholera vaccines in young children remain to be determined (16). We have shown in children receiving Dukoral (Crucell, Sweden), an oral killed whole-cell cholera vaccine containing recombinant CTB, that child vaccinees mount poor memory B cell responses at day 42 after vaccination, particularly for LPS, while children with clinical cholera infection develop better LPS-specific memory B cell responses (17). We also demonstrated that vaccinees <5 years of age were unable to generate significant memory T cell responses to cholera antigens, in contrast to older vaccinees (18). Long-term
immune responses following oral cholera vaccine administration in children have yet to be reported, and the magnitude and persistence of antibody avidity as measures of maturation of the immune response after vaccination have not been explored in children. Therefore, in this study, we sought to characterize the V. cholerae-specific antibody avidities of children who received an oral killed whole-cell cholera vaccine, to compare these responses with those of adult vaccinees, and to determine the relationship of antibody avidities over time to memory T cell responses shortly after vaccination.

MATERIALS AND METHODS
Study design and subject enrollment. We enrolled healthy young children (age, 3.5 to 5 years; n = 20), older children (age, 7 to 14 years; n = 20) (17), and adults (age, 20 to 45 years; n = 33) (11), who were given two doses of the oral killed whole-cell cholera vaccine Dakoral (Crucell, Sweden), with a 2-week interval. We obtained blood from children at the time of enrollment (day 0), 3 days after administration of the first dose of vaccine, and again on days 42 (28 days after the second dose), 90, 180, 270, and 360. For adults, we collected blood at the time of enrollment (day 0), 3 days after administration of each dose of vaccine (days 3 and 17), and again on days 42 (28 days after the second dose), 90, 180, 270, and 360. Of note, we previously reported nonavidity data for the aforementioned children through 42 days of follow-up, and we now extend these observations through 360 days of follow-up and correlate avidity data with other immune responses (17, 18). Similarly, we previously reported results for a subset of adults in the vaccinee cohort described in the current analysis (11), and we now supplement these results with data from additional adults, include avidity responses, and compare these results with those observed in children. In our current analysis, we include assessments of vibriobial antibodies, antigen (CTB and LPS)-specific antibody responses, and avidities of plasma antibodies for these antigens in both child and adult vaccinees. We assessed antigen-specific IgG and IgA memory B cell responses on days 0, 42, 90, 180, 270, and 360. Memory T cell responses were measured in children only on days 0, 21, and 42. All studies were approved by the research review and ethical review committees of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) (Dhaka, Bangladesh), and the institutional review board of the Massachusetts General Hospital.

Isolation of peripheral blood mononuclear cells and detection of memory B cell responses. We isolated peripheral blood mononuclear cells (PBMCs) and plasma from heparinized blood diluted with phosphate-buffered saline (PBS) by centrifugation on Ficoll-Isopaque (Pharmacia, Piscataway, NJ). Plasma was stored at −20°C for immunological assays. We resuspended PBMCs to a concentration of 1 × 10⁶ cells/ml in RPMI complete medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum, 200 U/ml of penicillin, 200 µg/ml of streptomycin, 100 µM 2-mercaptoethanol (19, 20). After incubation of the plates for 5 to 6 days at 37°C in a 5% CO₂ incubator, cells were harvested, washed, and plated onto 96-well nitrocellulose-bottomed plates precoated with either GM1 ganglioside (3 nM) followed by recombinant CTB (2.5 µg/ml), V. cholerae O1 Ogawa LPS (25 µg/ml), keyhole limpet hemocyanin (2.5 µg/ml, as negative control), or affinity-purified goat anti-human IgG or IgA antibodies (Jackson Laboratories, Bar Harbor, ME) at 1:1,000 dilution to detect CTB and LPS IgG or IgA, respectively, and plates underwent development as described above. After the addition of 1 M sulfuric acid to the plates, the optical density (OD) was measured at 492 nm. We used an OD between 0.5 and 2.00 for non-NaSCN-treated wells for each sample to calculate the avidity index (AI) as the ratio of the OD of NaSCN-treated wells to the OD of the untreated wells, as described previously (17, 22). For each ELISA plate, we used a positive-control specimen derived from pooled convalescent-phase sera from cholera patients (21).

Correlation analysis with T cell responses. We used the flow cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA) method to determine lymphoblast formation in response to antigenic stimulation and to measure memory T cell responses, as previously described (23, 24) and reported (18). We correlate our avidity results with previously reported CTB-specific effector memory T cell (TEM) and follicular helper T cell (Tfh) responses (18).

Statistical analyses. Comparisons of immune responses within and between groups were tested for significance using the Wilcoxon signed-rank test and the Mann-Whitney U test, respectively. We used Spearman’s correlation to assess the relationship between avidity indices and T cell responses. All reported P values are two sided, with P values of <0.05 being considered the threshold for statistical significance. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) and SigmaStat 3.1 (Systat Software, Inc., San Jose, CA).
RESULTS

Study population. In this study, we report results from 40 child and 33 adult vaccinees (Table 1). Among the child vaccinees, 20 were younger children (median age, 5 years) and 20 were older children (median age, 10 years). Eighty percent of the child vaccinees (young children, n = 16; older children, n = 16) completed follow-up, and 81% of the adults completed follow-up to day 360. The schedules for vaccination, blood collection, and immunological assays for child and adult vaccinees are shown in Fig. 1.

Vibriocidal and antigen-specific antibody responses in plasma. Baseline (day 0) vibriocidal antibody levels and CTB- and LPS-specific IgA antibody responses were comparable among the different age groups (Fig. 2 and 3). However, baseline CTB- and LPS-specific IgG antibody levels in both young and older children were significantly higher than those seen in adults (P < 0.05) (Fig. 3). Child (both young and older) and adult vaccinees showed significant increases in vibriocidal antibody responses (P < 0.05) (Fig. 2) and CTB- and LPS-specific IgG and IgA antibody responses within 3 days after vaccination (P < 0.05) (Fig. 3), as reported previously (11,17), compared to their respective baseline values. However, these responses persisted for longer periods of time in adult vaccinees than in child vaccinees (Fig. 2 and 3).

Vibriocidal and CTB-specific IgG antibody responses in adult vaccinees persisted up to day 270 (P < 0.05) (Fig. 2A). Bars, mean responses; error bars, standard errors of the mean. *, statistically significant differences (P < 0.05) from the baseline (day 0) titer.

CTB-specific avidity indices in child and adult vaccinees. The magnitudes of avidity indices for CTB-specific IgG and IgA antibodies in adults were significantly greater than those seen in young children (P < 0.05) at baseline (day 0) (Fig. 4). Adults also had a higher CTB-specific IgA antibody avidity index than did older children. The avidity index (AI) values (mean ± standard error) for CTB-specific IgG antibodies in young and older children at day 0 were 30% ± 1.7% and 36% ± 2.1%, respectively, which increased significantly at day 21 to 47% ± 3.8% and 58% ± 4.3%, respectively (Fig. 4A). The increase in avidity index over baseline persisted significantly up to day 360 for both age groups of children. As reported previously, the adult vaccinees also showed significant elevation in CTB-specific IgG avidity (15), although this elevation waned somewhat more quickly than in child vaccinees. CTB-specific IgA antibody avidity index values in young and older children increased significantly by day 3 and particularly by day 21 (AIs, 55% ± 4.8% and 61% ± 4.5%, respectively), compared to baseline (AIs, 31% ± 3.2% and 32% ± 2.4%, respectively) (P < 0.05) (Fig. 4B). These elevations in the avidity of CTB-specific IgA antibodies also persisted up to day 360 in both young and older children. The increased avidity index of CTB-specific IgA antibodies again waned somewhat more quickly in adult vaccinees than in child vaccinees. The magnitudes of the peak CTB-specific IgG and IgA antibody avidity indices after vaccination in different age groups were comparable.

LPS-specific avidity indices in child and adult vaccinees. We detected a significantly lower magnitude of LPS-specific IgG antibody avidity indices at day 0 in young and older children than in adults (P < 0.05) (Fig. 4). However, the magnitudes of the baseline avidity indices of LPS-specific IgA antibodies in different age groups were comparable. The mean antibody avidity indices for LPS-specific IgG at day 0 (prevaccination) in healthy young and older child vaccinees were 36% ± 1.8% and 37% ± 1.5%, respectively. These antibody avidity index values increased by day 21 (AIs, 45% ± 2.9% and 44% ± 2.8%, respectively) before returning to baseline levels by day 42 (Fig. 4C). Similarly, statistically significant increases in LPS-specific IgA antibody avidity indices in young and older child vaccinees were detected only on day 21 (AI = 52% ± 3.1% and 52% ± 3.6%, respectively), compared to

### TABLE 1 Demographic characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Young children</th>
<th>Older children</th>
<th>Adults</th>
</tr>
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<tbody>
<tr>
<td>No. of participants</td>
<td>20</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>No. (%) who completed follow-up to day 360</td>
<td>16 (80)</td>
<td>16 (80)</td>
<td>27 (81)</td>
</tr>
<tr>
<td>Age (median [25th to 75th percentile]) (yr)</td>
<td>5 (4.6–5)</td>
<td>10 (9.2–12.7)</td>
<td>32 (26–39)</td>
</tr>
<tr>
<td>Female (n [%])</td>
<td>9 (45)</td>
<td>10 (50)</td>
<td>13 (39)</td>
</tr>
</tbody>
</table>

*Immune responses* *Avidity index* *Memory B cell*
baseline (AIIs, 42% ± 1.9% and 41% ± 1.7%, respectively), and these returned to baseline levels by day 42 (Fig. 4D). In adult vaccinees, the increases in LPS-specific IgG or IgA antibody avidity indices were detected only at day 17 but returned to baseline levels by day 42, as reported previously (15). The magnitudes of the peak LPS-specific IgG or IgA antibody avidity indices in different age groups were comparable.

Antigen-specific memory B cell responses. We analyzed the memory B cell responses to CTB and LPS by combining all child vaccinees into one cohort (since we found no differences in memory B cell responses between young and older child vaccinees [data not shown] and we had a paucity of matched paired data points for statistical analyses), and we compared these responses with those of adult vaccinees (a portion of which were reported previously) (15). For both child and adult vaccinees, significant increases in CTB-specific IgG memory B cell responses were detected at day 42 (P < 0.05), compared to the respective baseline responses at day 0 (Fig. 5A). These memory B cell responses to CTB persisted slightly longer in adult vaccinees than in child vaccinees, but both responses returned to baseline levels by day 90 to 180. The adult vaccinees also developed significantly higher CTB-specific IgA memory B cell responses at any follow-up study day (Fig. 5B). We did not detect any significant increases in memory B cell responses to LPS (IgG or IgA isotype) in either child or adult vaccinees at any time point throughout the study period (Fig. 5C and D). In children, we also did not detect any correlation between the antibody avidity indices of CTB- or LPS-specific IgG or IgA antibodies at various time points after vaccination and CTB- or LPS-specific memory B cell numbers on the corresponding days (data not shown).

Correlation between early memory T cell responses and subsequent antibody avidity in older child vaccinees. To determine the relationship between memory T cell responses (specifically memory follicular helper T cell responses) and subsequent antibody avidities, we compared mutant cholera toxin-specific (18) effector memory T cell (TEM) and memory follicular helper T cell (TFH) responses at day 21 (7 days after the second dose of vaccine) with CTB-specific IgG or IgA antibody avidity indices in the same older child vaccinees on days 42, 90, 180, 270, and 360. We found that CTB-specific IgG antibody avidity indices on days 42, 90, 180, and 270 correlated with the memory T cell responses on day 21 (Spearman’s ρ = 0.45 to 0.65, P < 0.05) (Table 2); there was a trend toward a correlation at day 360 (Spearman’s ρ = 0.47, P = 0.08). The correlations between memory follicular helper T cell responses at day 21 and CTB-specific IgG antibody avidity indices on days 90, 180, and 270 were also statistically significant (Spearman’s ρ = 0.45 to 0.65, P < 0.05) (Table 2). We did not find a correlation between CTB-specific IgA antibody avidity indices at later time points and earlier memory T cell responses. We also did not find any correlations between T cell responses and avidity for the younger children.
DISCUSSION

Young children are vulnerable to cholera in both endemic and epidemic settings. Despite this, current oral cholera vaccines achieve lower protective efficacy and shorter duration of protection in young children than in older persons (6). Young children generally respond poorly to polysaccharide antigens (25–27). Previously, we demonstrated that memory B cell responses in young children to both the T cell-dependent antigen CTB and the T cell-independent antigen LPS following vaccination were significantly lower than those achieved after natural infection (17). Antibody avidity has been shown to be a correlate of immune memory and protective immunity in both infection and vaccination for other infectious diseases in children (28–31). In this study, we evaluated the magnitude and duration of antibody avidity in both children and adults who received two doses of oral cholera vaccine, with an interval of 2 weeks (day 0 and day 14). (A and C) IgG responses. (B and D) IgA responses. Bars, mean responses; error bars, standard errors of the mean. *, statistically significant differences (P < 0.05) from baseline (day 0) AI; #, statistically significant differences between age groups (P < 0.05).

FIG 4 Avidity index (AI) values for CTB-specific (A and B) and LPS-specific (C and D) antibodies in Bangladeshi vaccine recipients of different age groups who received two doses of oral cholera vaccine, with an interval of 2 weeks (day 0 and day 14). (A and C) IgG responses. (B and D) IgA responses. Bars, mean responses; error bars, standard errors of the mean. *, statistically significant differences (P < 0.05) from baseline (day 0) AI; #, statistically significant differences between age groups (P < 0.05).

Antibody avidity represents the combined binding affinities of a variety of antibodies and their multivalent antigen (15, 32). Class switching, affinity maturation, and somatic hypermutation that occur during B cell maturation generate high-affinity antibodies of different subclasses; this process is most efficient in the germinal centers (GCs) of secondary lymph nodes (33–35). During the primary immune response, CD4+ helper T cells differentiate into follicular helper T cells (Tfh) and interact with B cells in the GC, inducing antigen-specific B cell proliferation and maturation. The B cells that produce high-affinity antibodies during proliferation either differentiate into plasma cells or become memory B cells (33, 36). Previously, we have shown that adult cholera patients and vaccinees both develop high-avidity CTB- and LPS-specific antibodies and that these responses correlate well with memory B cell responses measured at the same time points, consistent with the idea that the two types of measurements reflect B cell maturation in similar manners (15). However, in this study, we did not find such correlation for children after two doses of Dukoral. We also showed previously that early T cell responses to CTB correlate with later memory B cell responses to the T cell-dependent antigen CTB in older children following vaccination but not in young children immunized with two doses of Dukoral (18). In this study, we found a significant positive correlation between early memory T cell responses (both TEM and memory Tfh cell responses) to
cholera toxin and subsequent CTB-specific IgG antibody avidity index values in older children who received vaccine but not young children. T cell help may function to stimulate affinity maturation of CTB-specific B cells in older children, while similar T cell help is not stimulated by vaccination in young children.

The precise mechanism of protective immunity against *V. cholerae* infection is still unknown. However, it has been shown that CTB- and LPS-specific IgA antibodies as well as LPS-specific IgG memory B cell responses are associated with decreased risk of subsequent infection (8,9). The memory B cell response is a key component of protective immunity that facilitates anamnestic responses. Both adults and children with cholera develop CTB- and LPS-specific memory B cell responses within 30 days after infection (10,37), and we have shown that the CTB-specific responses in adults persist for at least 1 year following cholera infection, while LPS-specific responses wane more quickly (10). In contrast, adults immunized with two doses of Dukoral achieve only a short duration of CTB-specific memory B cell responses (11), and both adults and children who receive vaccine have no measurable memory B cell responses against LPS (17). These results, combined with those described above, suggest that currently available oral cholera vaccines may not induce sufficient T cell help to induce longer-term memory B cell responses, particularly in younger children, in comparison with natural infection and that this may be an explanation for the lesser degree and duration of protection.

Recently, we observed that adult cholera patients and vaccinees were capable of developing high-avidity antibodies to both CTB and LPS; however, high-avidity antibodies to LPS did not persist past 17 days after vaccination and 180 days after infection (15). These results were consistent with LPS-specific memory B cell responses in the same groups. In this study, we have shown that both young and older children are able to develop high-avidity antibodies to CTB and LPS. In the case of CTB, these high-avidity antibodies remained at elevated levels over baseline levels to 180 to

TABLE 2 Correlation between cholera toxin-specific early T cell responses and later antibody avidity indices in older child vaccinees

<table>
<thead>
<tr>
<th>T cell response on day 21</th>
<th>Day of AI assessment</th>
<th>Spearman’s ρ</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector memory T cell</td>
<td>42</td>
<td>0.53</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.46</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.58</td>
<td>0.01</td>
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<tr>
<td></td>
<td>270</td>
<td>0.66</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>0.48</td>
<td>0.08</td>
</tr>
<tr>
<td>Memory follicular helper T cell</td>
<td>42</td>
<td>0.3</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.55</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.53</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>0.56</td>
<td>0.02</td>
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<tr>
<td></td>
<td>360</td>
<td>0.37</td>
<td>0.19</td>
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</table>
Antibody Avidity in Children after Cholera Vaccination

360 days, although CTB-specific antibody responses and memory B cell responses waned by 42 days following vaccination. This suggests that measuring antibody avidity indices for T-cell-dependent antigens following vaccination may provide evidence of longer-term immune maturation than measured by the current memory B cell assay, and this may explain why these vaccines provide protection against severe disease for 2 to 3 years following vaccination even in the absence of memory B cell responses (7). On the other hand, elevations in LPS-specific antibody avidity, although detectable shortly after vaccination in both children and adults, did not persist, and memory B cells did not develop. We hypothesize that such differences in the persistence of high-avidity antibodies for LPS and CTB are due to the T cell-dependent nature of the CTB antigen and the importance of T cell help in affinity maturation and the induction of B cell memory. Since immune responses to LPS appear to correlate with subsequent protective immunity, the failure of individuals to show evidence of T cell-independent B cell maturation for LPS following vaccination, measured as either increased antibody avidity indices or the development of LPS-specific memory B cells, may explain in part the shortened duration of protection following vaccination.

Animal models and human studies have suggested that affinity maturation and memory B cell development in the germinal center result in increases in antibody avidity (33). In studies of children receiving polysaccharide vaccines against Haemophilus influenzae and Streptococcus pneumoniae, it has been observed that high-avidity antibodies are more potent than low-avidity antibodies in bacterial assays (38, 39). However, while these findings suggest that antibody avidity may be a marker of protective immunity for at least some diseases, the association between these high-avidity antibodies and protection against disease has not been proven, and this association has not been investigated for infections at the mucosal surface. Thus, further studies are needed to determine the role of antibody avidity for both CTB and LPS in protection against V. cholerae infection.

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