Comparison of Memory B Cell, Antibody-Secreting Cell, and Plasma Antibody Responses in Young Children, Older Children, and Adults with Infection Caused by *Vibrio cholerae* O1 El Tor Ogawa in Bangladesh†


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Received 3 May 2011/Returned for modification 6 June 2011/Accepted 14 June 2011

Children bear a large component of the global burden of cholera. Despite this, little is known about immune responses to cholera in children, especially those under 5 years of age. Cholera vaccine studies have demonstrated lower long-term protective efficacy in young children than in older children and adults. Memory B cell (MBC) responses may correlate with duration of protection following infection and vaccination. Here we report a comparison of immune responses in young children (3 to 5 years of age; n = 17), older children (6 to 17 years of age; n = 17), and adults (18 to 60 years of age; n = 68) hospitalized with cholera in Dhaka, Bangladesh. We found that young children had lower baseline vibriocidal antibody titers and higher fold increases in titer between day 2 and day 7 than adults. Young children had higher baseline IgG plasma antibody levels to *Vibrio cholerae* antigens, although the magnitudes of responses at days 7 and 30 were similar across age groups. As a surrogate marker for mucosal immune responses, we assessed day 7 antibody-secreting cell (ASC) responses. These were comparable across age groups, although there was a trend for older age groups to have higher levels of lipopolysaccharide-specific IgA ASC responses. All age groups developed comparable MBC responses to *V. cholerae* lipopolysaccharide and cholera toxin B subunit at day 30. These findings suggest that young children are able to mount robust vibriocidal, plasma antibody, ASC, and MBC responses against *V. cholerae* O1, suggesting that under an optimal vaccination strategy, young children could achieve protective efficacy comparable to that induced in adults.

Cholera is an acute dehydration diarrheal disease caused predominantly by the *Vibrio cholerae* O1 or O139 serogroup. Globally, the vast majority of cholera is caused by *V. cholerae* O1. Cholera is endemic in over 50 countries and affects 3 to 5 million people each year, causing more than 100,000 deaths (2, 41). In areas of the world in which cholera is endemic, children under 5 years of age have the highest burden of disease (12, 31), and during cholera epidemics, children and adults are both at risk of dehydrating illness (21, 24, 34). In a rural area of Bangladesh in which cholera is endemic, 80% of the population had detectable vibriocidal antibodies by the age of 15 years (26). Similarly, in a recent observational study in urban Bangladesh, household contacts of patients with *V. cholerae* O1 infection who were ≤5 years of age had a significantly higher risk of developing infection than older family members in a 21-day observational period following identification of the index household case (15).

Despite the high global burden of cholera among children, vaccination of young children has shown lower protective efficacy than that achieved in adults, and the duration of protection has been shorter in children (3). In a field trial in Bangladesh of an oral killed cholera vaccine that contained whole-cell organisms together with cholera toxin B (CtxB) subunit (WC-BS), protective efficacy in children aged 2 to 5 years was 38% for the first 6 to 12 months, compared to 78% for those aged >5 years; furthermore, at a 3-year follow-up, protective efficacy had returned to baseline in young children, compared with a 40% efficacy for adults (8). However, in a recent trial of a different formulation of an oral killed whole-cell vaccine lacking CtxB (Shanchol; Shantha Biotechnics Ltd., India), in Kolkata, India, children aged 1.0 to 4.9 years and adults had comparable levels of protective efficacy during the 2-year period of observation (37). Currently, it is recommended that young children receive 3 doses of Dukoral, a WHO-prequalified cholera vaccine containing killed whole cells plus recombinant CtxB (Crucell,
rate of optical density change (mOD/min) and, to compare across plates, calculated ELISA units that represented the ratio of the test sample to a standard of pooled convalescent-phase sera present on each plate (16).

**ASC assay.** We assessed peripheral blood IgG and IgA ASC responses using an enzyme-linked immunosorbent spot (ELISPOT) procedure (14, 30). Briefly, we coated nitrocellulose-bottomed plates (Mahan-4550; Millipore, Bedford, MA) with LPS (2.5 μg/ml) and GM1 ganglioside (3 nM), followed by recombinant CtxB (2.5 μg/ml) or affinity-purified goat anti-human immunoglobulin (5 μg/ml; Jackson Immunology Research, West Grove, PA), to assess total IgG and IgA cells. Wells were coated with keyhole limpet hemocyanin (KLH, 2.5 μg/ml; Pierce Biotechnology, Rockford, IL) as a negative control. After wells were blocked with RPMI containing 10% FBS, we added freshly isolated PBMCs (5 × 10^5 PBMCs/well) to antigen-specific wells and 1 × 10^5 PBMCs/well to immunoglobulin-coated wells, which were then serially 10-fold diluted. After the plates were incubated for 3 h at 37°C in an incubator with 5% CO2, the plates were washed and alkaline phosphatase-conjugated goat anti-human IgG and HRP-conjugated IgA (Southern Biotech, Birmingham, AL) were added. We incubated plates overnight and developed them with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT; Sigma, St. Louis, MO) for IgG spots and 3-amin-9-ethylcarbazole (AEC) for IgA spots. We averaged independent counts visualized by two individuals using a stereomicroscope (blue spots indicating IgG and red spots indicating IgA on the same wells).

**MBC assay.** We assessed MBC responses as previously described (10, 14). Briefly, we plated freshly harvested cells in 24-well cell culture plates (BD Biosciences, San Jose, CA) at 5 × 10^4 PBMCs/well in RPMI 1640 medium containing 10% FBS, 2 mM l-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, and 50 μM beta-mercaptoethanol, with or without a stimulatory cocktail optimized to stimulate antigen-independent proliferation and differentiation of memory B cells into ASCs (10). This cocktail consists of the mitogens CpG oligonucleotide (6 μg/ml; Operon, Huntsville, AL), a 1/100,000 dilution of crude pokeweed mitogen extract, and a 1/10,000 dilution of fixed Staphylococcus aureus Cowan (Sigma, St. Louis, MO). We also tested a novel stimulatory cocktail recently published in the literature (38), consisting of 3 μg/ml CpG oligonucleotide, 20 ng/ml human recombinant B cell-activating factor (rBAFF, Peprotech), and 5 ng/ml recombinant interleukin-15 (rIL-15, Peprotech), on the PBMCs of 7 healthy controls and 8 cholera patients. We incubated plates at 37°C in an incubator with 5% CO2 for 6 days, after which we washed and evaluated MBC responses by using an ELISPOT format as described above. We placed 80% of cells from each well into CtxB- or LPS-coated ELISPOT plates for detection of antigen-specific IgG and IgA MBC responses and 20% of cells from each well in anti-human Ig-coated ELISPOT plates to assess total IgG and IgA MBC responses. We incubated these plates for 5 h at 37°C, washed them, and then incubated them overnight with horseradish peroxidase-conjugated mouse anti-human IgG and IgA (Hybridoma Reagent Laboratory, Baltimore, MD). We developed plates using AEC and averaged counts read by two independent readers. We expressed antigen-specific memory B cell responses as the percent-age of antigen-specific ELISPOT counts per total stimulated isotype-specific IgG or IgA ASCs. IgG and IgA MBCs in the KLH were considered as negative controls. For inclusion in analysis, we required sufficient stimulation of total antibody isotype-specific memory B cells (defined as ≥3-fold increase for stimulated versus non-stimulated wells), fewer than three antigen-specific cells in the corresponding sample's unstimulated wells, and fewer than three antigen-specific cells in the corresponding sample's KLH MBC assay. Application of these criteria resulted in the inclusion of approximately 90% of all data.

**RESULTS**

**Study population.** We enrolled a total of 17 young children (ages 3 to 5 years), 17 older children (ages 6 to 17 years), and 68 adults (ages 18 to 60 years). Baseline demographic, microbiological, and clinical data of each group are displayed in Table 1. There were no significant differences between age...
groups for any of the potential confounding demographic variables evaluated, including use of soap, place of defecation, source of drinking water, and blood group.

**Vibriocidal response.** We measured vibriocidal antibody responses to the homologous O1 Ogawa serotype on study days 2, 7, and 30. Almost all (99%) patients mounted a 4-fold increase in vibriocidal titer by day 7. Young children had lower baseline (day 2) titers than adults (P = 0.02) (Fig. 1A) and had a higher fold increase in titer by day 7 than adults (P = 0.04) (Fig. 1B). There were no significant differences in the vibriocidal antibody titers at convalescence among the various age groups (days 7 and 30).

**Antigen-specific antibody responses in plasma.** All age groups had significantly higher IgG and IgA antibody levels toCtxB and LPS at days 7 and 30 than at day 2 (Fig. 2). Younger children had higher baseline (day 2) plasma LPS (P = 0.05) and CtxB (P = 0.03) IgG levels than adults, although no significant differences between age groups were seen at days 7 and 30. The day 2 and day 7 antigen-specific IgA levels in plasma were comparable in all groups, although adults had higher LPS IgA responses on day 30 than the youngest group (P = 0.07). Younger children had a significantly higher number of total circulating IgA ASCs than adults for all days examined (Fig. 3E).

**Antigen-specific MBC responses.** We compared two published methods to stimulate and assess MBC responses (10, 38). We found no differences in either the total or the antigen-specific MBC IgA and IgG responses between the two stimulation methods in 8 patients and 7 healthy controls. We here report the results obtained using the method of Crotty et al. (10). We assessed CtxB- and LPS-specific IgG and IgA memory B cell responses on days 2 and 30 (Fig. 4A to D). Baseline (day 2) MBC levels were comparable across all age groups, antigens, and isotypes, with the exception that there was a

**TABLE 1. Demographic and clinical characteristics of study subjects**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value for group of age:</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3–5 yr (n = 17)</td>
<td>6–17 yr (n = 17)</td>
</tr>
<tr>
<td>No. of patients who completed 30-day follow-up</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Mean age, yr (range)</td>
<td>4.4 (3–5)</td>
<td>9.5 (6–14)</td>
</tr>
<tr>
<td>Mean duration of diarrhea prior to hospitalization, h (SD)</td>
<td>14 (15)</td>
<td>22 (18)</td>
</tr>
<tr>
<td>Mean total intravenous fluid received at hospital, ml/kg body wt (SD)</td>
<td>231 (166)</td>
<td>184 (135)</td>
</tr>
<tr>
<td>Mean duration of hospital stay, h (SD)</td>
<td>48 (28)</td>
<td>38 (18)</td>
</tr>
<tr>
<td>No. (%) of subjects who used antibiotics in week prior to hospitalization</td>
<td>5 (29)</td>
<td>4 (24)</td>
</tr>
</tbody>
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**FIG. 1.** (A) Median vibriocidal antibody titers by age group, with error bars representing interquartile ranges. An asterisk denotes a significant difference (P < 0.05) from the baseline (day 2) titer. (B) Mean fold changes in vibriocidal titer between days 2 and 7. Error bars represent standard errors.
FIG. 2. Mean plasma antibody responses by age group as measured by ELISA, with error bars representing standard errors of the means. An asterisk denotes a significant difference ($P < 0.05$) from the baseline (day 2).
FIG. 3. (A to D) Mean absolute numbers of antigen-specific ASC responses by age group, as measured by ELISPOT assay, per $5 \times 10^6$ PBMCs, with error bars representing standard errors of the means. An asterisk denotes a significant difference ($P < 0.05$) from the baseline (day 2).

(E) Mean numbers of total ASC IgA responses by age group. Error bars represent standard errors.
FIG. 4. (A to D) Mean antigen-specific memory B cell responses by age group, as measured by ELISPOT assay after 6-day polyclonal stimulation, as percentage of total memory B cells, with error bars representing standard errors of the mean. (E) Mean numbers of total stimulated MBCs on day 30 by age group. Error bars represent standard errors.
trend for baseline IgA anti-CtxB MBC levels to be higher in adults than in children ($P = 0.08$ for adults versus older children). Importantly, we detected MBC responses on day 30 in all age groups, for both antigens and for both antibody isotypes. IgA MBC responses were comparable across all age groups. The CtxB IgG MBC response at day 30 tended to be higher in young children than in older children ($P = 0.06$). The LPS IgG MBC responses at day 30 were similar across all age groups. Following stimulation, infected adults had a larger total MBC IgG response than infected younger ($P < 0.0001$) and older ($P = 0.04$) children on day 30 (Fig. 4E). This pattern was also observed with the total MBC IgA response but to a lesser degree that was not significant ($P = 0.06$ for older children versus adults).

**DISCUSSION**

Although children bear a significant component of the global burden of cholera, relatively little is known about immune responses in children with cholera (7), especially in children less than 5 years of age. In this study, we compared immune responses in young children, older children, and adults with cholera in an area of cholera endemicity. The virobioidal antibody response is one of the most commonly used markers for assessing protection against cholera. It is thought to be a surrogate marker of protection since it detects the presence of complement binding anti-\textit{V. cholerae} antibodies in the peripheral blood. In areas of the world in which cholera is endemic, the mean virobioidal antibody titer increases with increasing age, presumably reflecting previous exposure to \textit{V. cholerae} (13). However, there is no level of virobioidal antibody titer absolutely associated with protection against cholera (32), and we and others have previously shown that virobioidal responses decline to baseline within 6 to 12 months after an episode of severe cholera in Bangladesh (14). This is despite the fact that protection against cholera exists for at least 3 years (the last period examined) following experimental exposure and rechallenge with the organism (22), and mathematical modeling suggests that previous infection with \textit{V. cholerae} affords significant protection that lasts up to 10 years (20). In the present study, we found that young children had the lowest baseline virobioidal antibody levels, presumably a reflection of the fact that older children and adults may have already been exposed to \textit{V. cholerae} infections. Despite this, the magnitudes of the virobioidal antibody response at convalescence were equivalent in all age groups, including younger children, showing that young children are capable of mounting robust virobioidal antibodies after exposure.

The virobioidal response is largely directed against LPS (27), and we have recently demonstrated that LPS IgA responses, as well as CtxB IgA responses, also correlate with protection against cholera (15). In our present study, we showed that children had higher CtxB IgG levels in peripheral blood at baseline than adults, possibly reflecting the presence of cross-reactive antibodies that recognize both cholera toxin (CT) and the heat-labile toxin (LT) of enterotoxigenic \textit{Escherichia coli} (ETEC) (13, 36), the latter a common cause of acute watery diarrhea in young children in resource-limited areas of the world (29). Despite these different baseline levels, all age groups had comparable CtxB IgA and IgG responses at convalescence. Baseline LPS IgG levels in blood were also slightly higher in children than adults, but the cause of this small difference is uncertain; however, the magnitudes of LPS-specific IgG responses in plasma at convalescence were comparable across all age groups. Baseline IgM responses to LPS in children were significantly higher than those in adults, which is opposite what was observed for virobioidal antibody. Despite the fact that the virobioidal antibody is mostly IgM directed to LPS (23, 25), we have previously observed that no correlation exists between plasma IgM and virobioidal antibodies in adults with cholera (18). The LPS-specific IgA response in adults was higher than that in young children on day 30; this observation may reflect the ability of adults to mount more-prominent immune responses to T cell-independent antigens than young children (19). In summary, despite some differences in immune responses, young children were able to mount prominent anti-tetoxin and antibacterial B cell responses in blood, and the magnitudes were comparable to those observed in older children and adults.

\textit{V. cholerae} is a noninvasive mucosal pathogen. To assess mucosal immune responses, we measured ASC responses in peripheral blood. Following mucosal stimulation, intestinal lymphocytes transiently migrate in the peripheral circulation before rehoming to intestinal tissue (11). This migration peaks 7 to 10 days after mucosal challenge. We found that young children were able to mount prominent anti-CtxB ASC responses that peaked on day 7 and that these responses were comparable to those observed in older children and adults. There was a trend toward older age groups having a higher day 7 LPS-specific IgA ASC response than the youngest group, which corresponded with the higher plasma LPS IgA response on day 30 in adults that we observed. These observations may reflect a lower mucosal response to T cell-independent antigens, such as LPS, in young children (9), and these responses may increase with age due to immune maturation and continued pathogen exposure. Interestingly, younger children had a higher total pool of IgA-producing ASCs in peripheral blood than adults, which corresponds with observations that the number of plasma cells is also inversely correlated with age (5).

We have previously shown that memory B cell responses to \textit{V. cholerae} O1 are detectable for at least 1 year after infection in adults and persist even after plasma antibodies and ASCs have returned to baseline levels (14). We were thus particularly interested in evaluating MBC responses in children compared to those in adults. In our current study, we found that both younger and older children were able to mount CtxB- and LPS-specific IgG and IgA MBC responses at day 30 that were comparable to those observed in adults. There was a trend for younger children to mount higher levels of CtxB IgG MBC responses than older children, perhaps reflecting more-recent exposure to the LT antigen of ETEC in young children. In comparison, there was a nonsignificant trend for adults to have higher baseline CtxB IgA MBCs. The cause and significance of this observation are uncertain, although the trend could reflect more-recent exposure to \textit{V. cholerae}.

We also found that the total stimulated IgG MBCs increased with age, a phenomenon previously reported in a population from a region of malaria endemicity (39). A similar trend was noted for the IgA MBC compartment. These findings may be expected, as circulating MBCs are likely a reflection of a cu-
mulative lifetime of antigenic exposures. When the absolute numbers of antigen-specific MBC spots per million PBMCs were measured, adults had a higher baseline LPS IgA MBC level than young children, which may reflect previous exposure; however, when this measurement was calculated as a percentage of total MBCs, this trend disappeared, given the overall higher number of MBCs among PBMCs in adults.

A limitation to our study is that due to difficulties inherent in enrollment of young children, the sample size of the groups of children in our study is relatively small compared to that of our adult cohort. In addition, in this study we followed children only up to 30 days postinfection. The persistence of the MBC response in young children, compared to that seen in adults (14), may further reveal the mechanism behind differences in vaccine efficacy, and a study addressing this issue is ongoing.

Current cholera immunization strategies focus on oral, whole-cell killed and live attenuated vaccines. These vaccines are protective, but their duration of protection is less than that afforded by wild-type infection, even in adults (20, 35). The immunogenicity of protection appears to be of a shorter duration in young children than in adult vaccine recipients (35, 37), despite the fact that children under 5 years of age also bear a large burden of cholera in areas of the world in which cholera is endemic (12). Natural cholera infection includes exposure to cholera toxin, which is not only an enterotoxin but also a potent immunoadjuvant (33). We have recently analyzed memory B cell responses in adult recipients of an oral cholera vaccine in Bangladesh and noted that responses were both less prominent and of shorter duration than those induced by wild-type disease (1). We hypothesize that these reduced responses following vaccination compared to infection may reflect the absence of the immunoadjuvantive properties of CT in current oral cholera vaccines. Experimental studies in animal models evaluating oral adjuvanticity of the toxin have demonstrated that CT stimulates a greater mucosal immune response than CtxB alone (4, 6); unfortunately, the use of CT as a mucosal adjuvant is limited by the fact that children under 5 years of age also bear a large burden of cholera in areas of the world in which cholera is endemic (12).

ACKNOWLEDGMENTS

This work was supported by the ICDDR,B and grants from the National Institutes of Health, including the National Institute of Allergy & Infectious Diseases (U01 AI058935 [S.B.C. and E.T.R.], R03 AI063079 [F.Q.], and U01 AI077883 [E.T.R.]); a Training Grant in Vaccine Development from the Fogarty International Center (TW05572 [M.M.A., M.S.B., and F.Q.]); an American Recovery and Reinvestment Act (ARRA) FIC Postdoctoral Fellowship in Global Infectious Diseases (TW05572 [D.T.L.]); Career Development awards (K01) from the Fogarty International Center (TW007409 [J.B.H.] and TW07144 [R.C.L.]), the Infectious Diseases Society of America Med-


