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Study of Avidity of Antigen-Specific Antibody as a Means of Understanding Development of Long-Term Immunological Memory after *Vibrio cholerae* O1 Infection

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The avidity of antibodies to specific antigens and the relationship of avidity to memory B cell responses to these antigens have not been studied in patients with cholera or those receiving oral cholera vaccines. We measured the avidity of antibodies to cholera toxin B subunit (CTB) and *Vibrio cholerae* O1 lipopolysaccharide (LPS) in Bangladeshi adult cholera patients ($n = 30$), as well as vaccinees ($n = 30$) after administration of two doses of a killed oral cholera vaccine. We assessed antibody and memory B cell responses at the acute stage in patients or prior to vaccination in vaccinees and then in follow-up over a year. Both patients and vaccinees mounted CTB-specific IgG and IgA antibodies of high avidity. Patients showed longer persistence of these antibodies than vaccinees, with persistence lasting in patients up to day 270 to 360. The avidity of LPS-specific IgG and IgA antibodies in patients remained elevated up to 180 days of follow-up. Vaccinees mounted highly avid LPS-specific antibodies at day 17 (3 days after the second dose of vaccine), but the avidity waned rapidly to baseline by 30 days. We examined the correlation between antigen-specific memory B cell responses and avidity indices for both antigens. We found that numbers of CTB- and LPS-specific memory B cells significantly correlated with the avidity indices of the corresponding antibodies ($P < 0.05$; Spearman's $\rho = 0.28$ to 0.45). These findings suggest that antibody avidity after infection and immunization is a good correlate of the development and maintenance of memory B cell responses to *Vibrio cholerae* O1 antigens.

Vibrio cholerae O1 can cause acute watery diarrhea with severe dehydration in both children and adults and may lead to death if untreated (1). Natural infection with *Vibrio cholerae* O1 induces protection against subsequent symptomatic disease for at least 3 to 7 years (2, 3). Considerable efforts have been made in the last 3 decades to develop safe and effective oral cholera vaccines. However, the currently available licensed vaccines give protection for between 2 to 3 years, shorter than that following natural infection (4, 5). Several markers of protection against cholera on exposure, such as plasma IgA against *V. cholerae* O1 lipopolysaccharide (LPS) (6), vibriocidal antibodies (7, 8), and memory B cell responses to cholera antigens (9, 10), have been suggested as potential markers of protective immunity. However, plasma antibody responses decrease in the 6 to 9 months after cholera, before protective immunity wanes, and while memory B cell responses persist longer, assay of these responses is more complicated. It would be helpful in understanding protective immunity to cholera after infection or vaccination if there was an earlier indicator of subsequent development of longer-term immunological memory.

In previous studies with patients, we have shown that robust systemic and mucosal antibodies against lipopolysaccharide (LPS), cholera toxin B subunit (CTB), and colonization factors, including the major subunit of the toxin-coregulated pilus (TcpA), develop after cholera and these may play a role in protection against subsequent infection (11–14). Responses against protein antigens were detectable for a longer period than responses to the T cell-independent carbohydrate antigen LPS, suggesting that T cells may be important for developing and maintaining memory B cell responses (15).

Antibody avidity, the functional affinity of multivalent antibody to bind multivalent antigen, has been used as a marker of B cell maturation to discriminate between primary and secondary responses to a number of infections, including infections with dengue virus (16), rubella virus (17), (18), cytomegalovirus (19), and herpesvirus (20). Furthermore, antibody avidity is an important surrogate for determining protective immunity for several vaccines, including measles vaccine (21), *Haemophilus influenzae* type b conjugate vaccine (22), pneumococcal capsular polysaccharide vaccine (23), and mumps vaccine (24). However, studies on avidity of antibodies generated following cholera and cholera vaccination have not been reported to date, nor has this been assessed for other noninvasive infections of the gastrointestinal

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TABLE 1 Characteristics of study participants

Characteristic	Value for group[a]	
	Cholera patients ($n = 30$)	Vaccinees ($n = 30$)
Median age, yr (range)	31 (18–46)	32 (20–45)
Sex, no. (%) of subjects		
Male	17 (57)	17 (57)
Female	13 (43)	13 (43)
ABO blood group, no. (%) of subjects		
A	7 (23)	7 (23)
B	11 (37)	10 (33)
AB	1 (3)	1 (3)
O	11 (37)	12 (40)
Infecting *Vibrio cholerae* O1 serotype, no.		
Ogawa	20	NA[b]
Inaba	10	NA

[a] There were no significant differences between the study groups.
[b] NA, not applicable.

tract. It is also not known if the avidity of antibody responses correlates with the development and duration of memory B cell responses.

In this study, we analyzed the avidity of IgG and IgA antibodies to both a T cell-dependent antigen (CTB) and a T cell-independent antigen (LPS) following natural cholera infection and oral cholera vaccination, and we correlated these with the development of memory B cell responses recognizing the same antigens.

MATERIALS AND METHODS

Study subjects and specimens. We enrolled adult cholera patients presenting to the hospital of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) (9). We also enrolled healthy adults from an urban field site in Mirpur, Dhaka, Bangladesh, who were given two doses of the inactivated whole-cell oral cholera vaccine Dukoral at 2-week intervals (25). The healthy volunteers did not take antibiotics in the past 2 weeks, which was a criterion for enrollment into the study. Venous blood was collected at the time of enrollment (day 2 for patients and day 0 for vaccinees) and 3 days after intake of each dose of vaccine; follow-up blood samples were obtained from patients and vaccinees on study days 30, 90, 180, 270, and 360 to measure antibody avidity responses by enzyme-linked immunosorbent assay (ELISA). Specimens from both the patients with cholera and following cholera vaccination (Table 1) were used for plasma antibody avidity measurements and for analyses of memory B cell responses to two key antigens, CTB and LPS (25). The specimens for this analysis were obtained from Institutional Review Board (IRB)-approved studies of the Massachusetts General Hospital and/or ICDDR,B.

Isolation of PBMCs. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from heparinized blood diluted with phosphate-buffered saline (PBS) by centrifugation on Ficoll-Isopaque (Pharmacia). Isolated PBMCs were resuspended to a concentration of 1×10^7 cells/ml in RPMI complete medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (10) and were used for the memory B cell culture. Plasma was frozen at −70°C for serologic assays.

CTB- and LPS-specific antibodies in patients and vaccinees. Both the IgG and IgA antibody responses were measured by ELISA using previously described procedures (14, 26). We used 96-well polystyrene plates (Nunc F; Nunc, Denmark) coated with *V. cholerae* O1 Ogawa LPS for the vaccinees (250 ng/well) and the serotype of *V. cholerae* O1 LPS (Inaba or Ogawa) homologous to their infecting strain for patients. For the toxin-specific responses, plates were coated with ganglioside GM1 (0.3 nmol/ml), followed by recombinant CTB subunit (50 ng/well) (both gifts from A. M. Svennerholm, University of Gothenburg, Gothenburg, Sweden). Then, 100 μl of plasma (diluted 1:100 for CTB and 1:25 for LPS in 0.1% bovine serum albumin in phosphate-buffered saline–Tween) was added per well, followed by horseradish peroxidase-conjugated secondary antibodies to human IgG or IgA (Jackson ImmunoResearch, West Grove, PA). Plates were developed with *ortho*-phenylene diamine (Sigma, St. Louis, MO) in 0.1 M sodium citrate buffer (pH 4.5) and 0.1% hydrogen peroxide. Each plate was read kinetically at 450 nm for 5 min; the maximal rate of change for samples in optical density in milli-absorbance units per minute was normalized across plates by calculating the ratio of the test sample value to a standard of pooled convalescent-phase serum from previously infected cholera patients included on each plate.

Plasma CTB- and LPS-specific IgG and IgA antibody avidity measured by ELISA. Avidity indices for CTB- and LPS-specific plasma IgG and IgA antibody responses were measured as described earlier (27). Briefly, 96-well microtiter plates (Nunc F) were coated as described above. The plates were incubated with two pairs of serially diluted plasma samples (1:5 to 1:2,000 dilution in 0.1% bovine serum albumin–phosphate-buffered saline–Tween) for 90 min at room temperature (RT). The supernatants of the wells were discarded, and one pair of the wells for each sample was treated with a chaotropic agent, sodium thiocyanate (NaSCN) (2 M in PBS–0.3% Tween) for 20 min at RT, whereas the other pair of wells was treated with PBS–0.3% Tween alone. After washing with PBS-Tween, horseradish peroxidase-conjugated anti-human IgG or IgA antibodies (Jackson Laboratories, Bar Harbor, ME) were added at a 1:1,000 dilution, and plates were developed as described above. The optical density (OD) was measured at 492 nm after addition of 1 M sulfuric acid to the plates. For each patient sample, non-NaSCN-treated wells with an OD of between 0.5 and 2.0 were used for the calculation of antibody avidity (28). The avidity index (AI) was calculated as the percentage of the ratio of measured optical density (OD) in the NaSCN-treated wells to that in the untreated wells (27) (AI [%] = OD of NaSCN-treated well × 100/OD of non-NaSCN-treated well). A positive-control specimen derived from pooled, convalescent-phase sera from cholera patients was included on each ELISA plate (25, 26).

Memory B cell culture and ELISPOT assay. We assessed memory B cell responses at study initiation (days 0 and 2 in vaccinees and patients, respectively) and on days 30, 90, 180, 270, and 360 using a previously described method (9, 10, 29, 30). For this assay, 5×10^5 cells/well were cultured in 24-well cell culture plates (BD Biosciences, San Jose, CA) containing culture medium optimized to stimulate antigen-independent proliferation and differentiation of memory B cells into antibody-secreting cells (ASCs). The medium consisted of RPMI 1640, 10% FBS, 200 U of penicillin/ml, 200 g of streptomycin/ml, 2 mM L-glutamine, 50 M β-mercaptoethanol, and a mixture of three B cell mitogens, i.e., CpG oligonucleotide (Operon, Huntsville, AL), crude pokeweed mitogen extract, and fixed *Staphylococcus aureus* Cowan (Sigma). As a negative control, PBMCs were also placed into wells containing this culture medium lacking mitogens. The plates were incubated at 37°C in a 5% CO_2 incubator for 5 to 6 days, after which the cells were harvested and used to perform antigen-specific (CTB and LPS) and total IgG and IgA enzyme-linked immunospot (ELISPOT) assays. IgG and IgA ASCs were detected using horseradish peroxidase-conjugated mouse anti-human IgG and IgA (Hybridoma Reagent Laboratory), respectively, followed by development with 3-amino-9-ethyl carbazole (AEC). ELISPOT counts were expressed as the percentage of antigen-specific memory B cells out of the total IgG or IgA memory B cells in the culture. Wells coated with keyhole limpet hemocyanin (KLH) (Pierce Biotechnology, Rockford, IL) were used as a negative control. Criteria for inclusion and exclusion of data for analyses were as described previously (25).

Statistical analyses. Comparisons of the immune responses during the 1-year follow-up were tested for significance within different study groups using the Wilcoxon signed rank test and between groups using

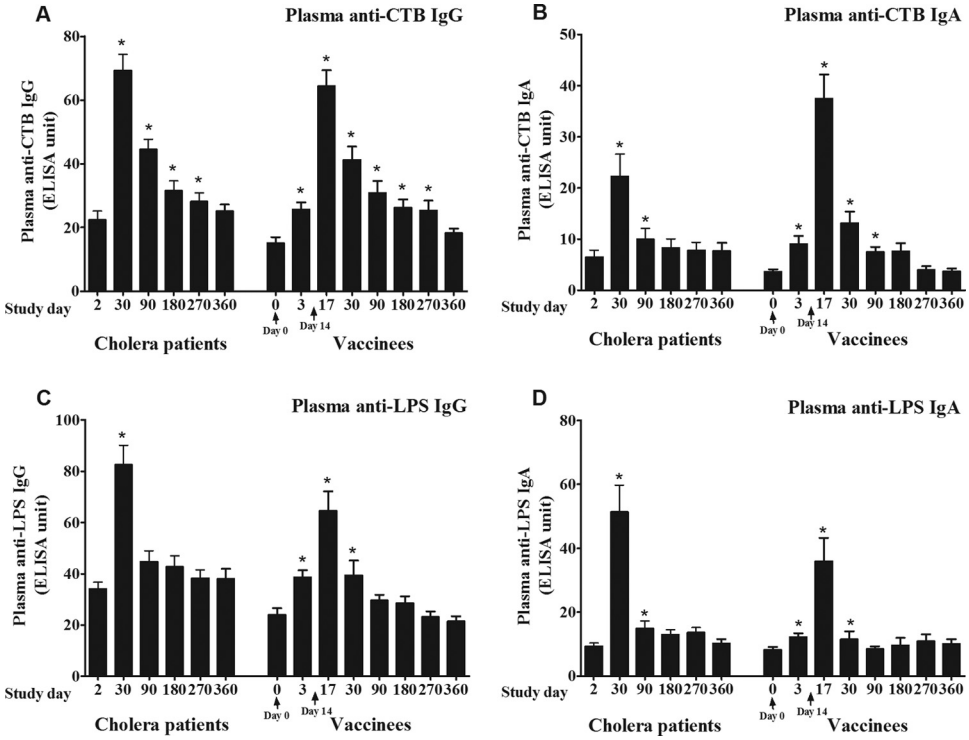

FIG 1 CTB-specific (A and B) and LPS-specific (C and D) antibody responses in plasma in adult Bangladeshi cholera patients and in vaccine recipients who received two doses of cholera vaccine at an interval of 2 weeks apart (day 0 and day 14, indicated by arrows). Bars indicate mean responses, and error bars represent standard errors of the means. The Wilcoxon signed rank test was used for analyses of data. *, statistically significant difference ($P < 0.05$) from the baseline (day 0 or day 2) titer and the follow-up days indicated.

the Mann-Whitney U test. We also used repeated-measures multivariate analysis of variance (MANOVA) to determine differences between groups. All reported *P* values are two sided, with a *P* value of <0.05 considered a threshold for statistical significance. Spearman's correlation analysis was used to measure bivariate associations. Analyses were performed using GraphPad Prism 5.0, SigmaStat 3.1, SPSS 14.0, and STATA 10.

RESULTS

Study population. Patients admitted to the ICDDR,B hospital with dehydration were treated with intravenous fluid as well as with azithromycin. For the patients, the mean duration of stay in the hospital was 38 h. We enrolled 30 adult cholera patients, both males (57%) and females (43%), for plasma antibody avidity measurements and for analyses of correlation of avidity with memory B cell responses to CTB and LPS. Specimens from 30 healthy adult cholera vaccinees (57% male and 43% female) were also analyzed. Vaccinees were disease free at the time of specimen collection. However, during the course of the study we did not follow them up for illnesses; they were also not admitted to the ICDDR,B diarrheal hospital for any disease during the study period. Details of the patients and vaccinees are shown in Table 1; the demographic characteristics of the patients and vaccinees in the study were comparable. All strains of *V. cholerae* O1 were of the El Tor biotype and of the altered variant and of both Ogawa and Inaba serotypes. There were no differences in the severity of diarrhea between cholera patients infected with the Ogawa or Inaba serotype of *V. cholerae* O1.

CTB- and LPS-specific IgG and IgA antibodies in patients and vaccinees. An antibody response at any time point was defined as a significantly higher response than day 2 or day 0 responses for patients and vaccinees, respectively, and both Ogawa- and Inaba-infected patients showed comparable responses ($P > 0.05$). Responses to both CTB and LPS were similar in patients and vaccinees, although the anti-LPS-specific IgA responses waned earlier in vaccinees (Fig. 1). The CTB-specific responses lasted longer than the LPS-specific responses in both patients and vaccinees. This is probably because the response to the protein antigen CTB is T cell dependent whereas that to LPS is T cell independent, resulting in an immune response of shorter duration. The IgG responses were of higher magnitude than the IgA responses. Antibody responses peaked at day 30 in patients. In vaccinees, significant responses were found on day 3, after the first dose of vaccine (day 0), and these were substantially boosted at day 17, that is 3 days after intake of the second dose of vaccine (given on day 14), showing preexistence of CTB- and LPS-specific memory B cells in the study individuals.

Avidity of CTB-specific antibodies in patients and in vaccinees. The mean CTB-specific IgG antibody avidity index (AI) in cholera patients on day 2 of infection was 35% ± 1.2%; this increased significantly at day 30 (AI = 48% ± 2.8%, $P = 0.0002$) and remained elevated throughout the 360 days of follow-up (Fig. 2A). Prior to vaccination, the CTB-specific IgG AI in healthy adults was 37% ± 1.6%, similar to that in the acute stage in patients. There was no change in the avidity index at 3 days after intake of the first dose of vaccine, but the AI increased significantly by day 17 (60% ± 2.8%), after the second dose of vaccine; the AI decreased gradually and reached baseline levels within 270 days of follow-up. The CTB-specific IgG AI in patients was significantly higher than

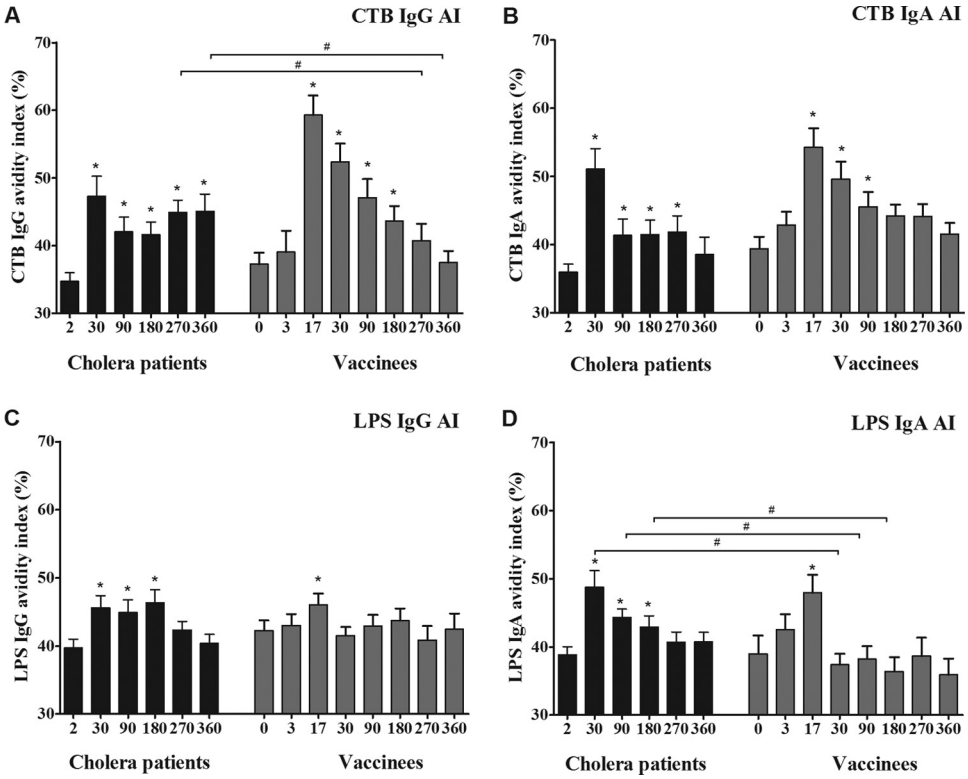

FIG 2 Avidity indices of CTB-specific (A and B) and LPS-specific (C and D) antibodies in plasma in Bangladeshi adult cholera patients and in vaccine recipients who received two doses of cholera vaccine at an interval of 2 weeks (day 0 and day 14). Bars indicate mean responses, and error bars represent standard errors of the means. The Wilcoxon signed rank test was used for analyses of data. *, statistically significant difference ($P < 0.05$) from the baseline (day 0 or day 2) titer to follow-up indicated above; #, statistically significant difference between cholera patients and vaccinees ($P < 0.05$).

that in vaccinees at days 270 and 360. Similarly, the CTB-specific IgA AI remained elevated in patients up to day 270 of follow up (AI = 42% ± 2.3%; $P = 0.0352$), while the AI in vaccinees remained significantly elevated only out to 90 days after vaccination (AI = 46% ± 2.2%; $P = 0.0135$) (Fig. 2B). Comparable responses in the CTB-specific IgA were seen at each time point of analysis up to day 360 (Mann-Whitney U test). However, significant differences in CTB responses were seen between the two groups ($P < 0.001$) using repeated-measures MANOVA.

LPS-specific avidity responses in patients and vaccinees. On day 2 of infection, the LPS-specific IgA AI was approximately 39% in patients; this increased significantly at day 30 (AI = 49% ± 2.4%; $P = 0.0005$) and remained elevated up to day 180 postinfection before decreasing to baseline levels (Fig. 2D). The prevaccination LPS-specific IgA AI in healthy adults was 39% (day 0), similar to that at day 2 in patients. A significantly higher LPS-specific IgA AI (AI = 48% ± 2.6%; $P = 0.0168$) was detectable only on day 17 in vaccinees but returned to baseline levels by day 30 and beyond (Fig. 2D). The LPS-specific IgA AI was significantly higher in patients with cholera for up to 180 days following infection than the AI following two doses of vaccination in healthy Bangladeshi adults. A similar pattern of AIs was observed for LPS-specific IgG. The AI remained elevated up to 180 days following infection (AI = 46% ± 1.9%; $P = 0.0014$) but was elevated only at day 17 in vaccinees (Fig. 2C). Compared to that in vaccinees, the magnitudes of the LPS-specific IgG or IgA AI in patients at days 270 and 360 were comparable, and they waned down to baseline on these study days. Using MANOVA, a difference in the IgA but not the IgG response was seen.

Antigen-specific IgG and IgA memory B cell responses. Statistically significant increases in CTB-specific IgG memory B cell responses compared to the baseline responses were detected at day 30 in both cholera patients and vaccinees ($P < 0.05$) (Fig. 3). The CTB-specific IgA memory responses were also elevated by day 90 and day 30 in patients and vaccinees, respectively. The memory B cell response in patients to CTB-specific IgA on day 30 was increased, but this did not reach statistical significance ($P = 0.057$), probably due to the lower numbers of matched data points that were available for this analysis. In patients, CTB-specific IgG memory B cell responses persisted for up to 1 year, while the response waned by day 270 in vaccinees. An anti-CTB-specific IgA memory B cell response could also be seen at the later stage at day 270 in patients but not in vaccinees. Cholera patients also developed LPS-specific IgA and IgG memory B cell responses that were significantly elevated by day 30 and day 180, respectively, although these levels decreased by 360 days (Fig. 3). In comparison, we did not detect any significant increases of LPS-specific IgA or IgG memory B cell responses in the vaccine recipients at any time point.

Correlation between antibody avidity and antigen-specific memory B cell responses. To determine the correlation between antibody avidity index and memory B cell responses, we compared avidity indices with memory B cell responses on corresponding days for each antigen and each antibody isotype in both

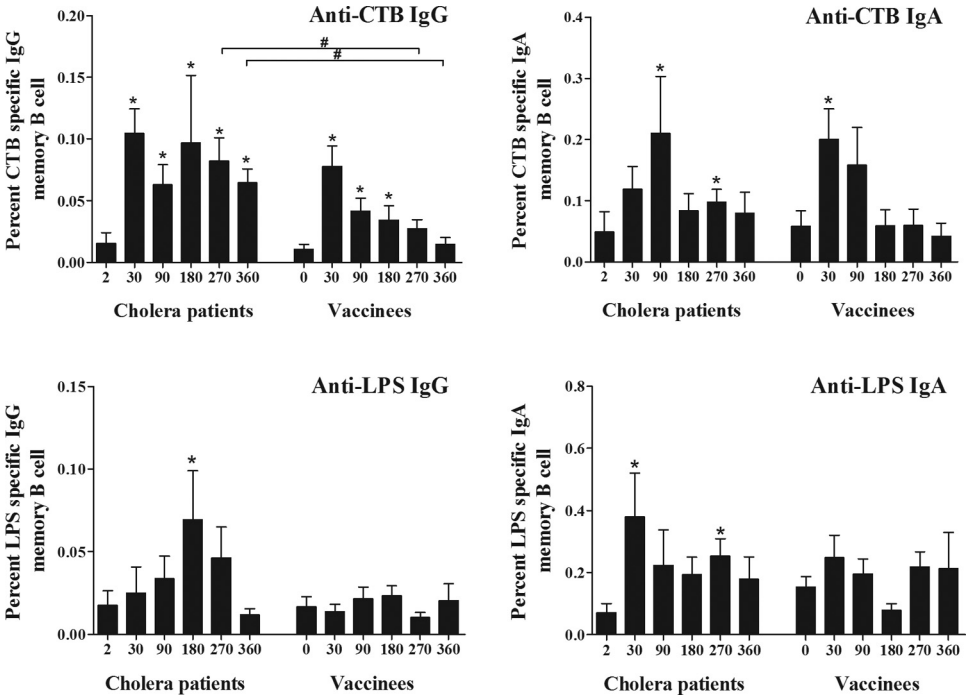

FIG 3 CTB and LPS memory B cell responses in Bangladeshi adult cholera patients and vaccinees who received two doses of cholera vaccine separated by 2 weeks (day 0 and day 14). Memory B cell responses are expressed as percent antigen-specific responses of total isotype-specific memory B cells. Bars represent mean responses, and error bars represent standard errors of the means. *, statistically significant difference ($P < 0.05$) from the baseline (day 0 or day 2) titer; #, statistically significant difference between cholera patients and vaccinees ($P < 0.05$).

patients and vaccinees. Both CTB- and LPS-specific IgG and IgA avidity indices in patients and vaccinees showed significant positive correlations with corresponding memory B cell responses (Spearman's $\rho = 0.28$ to 0.45; $P < 0.05$) (Table 2).

DISCUSSION

In this study, we have shown that antibody responses following cholera and cholera vaccination are robust and develop significant avidity for both the T cell-dependent antigen cholera toxin and the T cell-independent antigen lipopolysaccharide. This is the first study that we are aware of that assesses antibody avidity following a noninvasive mucosal infection, and we show that not only IgG but also IgA antibodies develop significant avidity. We also show that antibody avidity measurements correlate with memory B cell responses to the same antigen at the same time point, consistent with both of these responses developing in parallel during B cell maturation following antigen exposure.

Memory B cells are a major component of antibody-mediated long-term protective immunity following infection or vaccination. For T cell-dependent antigens, memory B cell development and differentiation occur in germinal centers (GC) of secondary lymphoid tissue (31). Upon exposure to an antigen, antigen-presenting cells such as dendritic cells present epitopes of that antigen to $CD4^+$ helper T cells, leading to the differentiation of these CD4 T cells into T follicular helper cells (Tfh), which migrate into the GC of intestinal Peyer's patches and mesenteric lymph nodes. These Tfh, characterized by expression of CXCR5, interact in GC with B cells that recognize that epitope, leading to proliferation of the B cell with somatic hypermutation and isotype switching. One result of this proliferation and maturation is the production of plasma cells (both short- and long-lived) of higher antibody affinity after negative selection in the GC of low-affinity antibody-producing B cells (31, 32). The other result of this proliferation and maturation process is the generation of memory B cells that can play a role in subsequent protective immunity (29). We have recently shown that early T cell responses to CTB following either cholera or cholera vaccination correlate well with subsequent memory B cell responses, consistent with the sequential events outlined above for maturation of the B cell responses for T cell-dependent antigens (33). Generation of highly avid IgG antibodies

TABLE 2 Correlation analyses of antibody avidity index and memory B cell responses in patients and vaccinees[a]

Comparison[b]	Spearman's ρ	*P* value
CTB specific		
IgG AI and IgG MBC		
Patients	0.350	<0.001
Vaccinees	0.450	<0.001
IgA AI and IgA MBC		
Patients	0.298	0.012
Vaccinees	0.376	<0.001
LPS specific		
IgG AI and IgG MBC		
Patients	0.369	0.006
Vaccinees	0.373	<0.001
IgA AI and IgA MBC		
Patients	0.283	0.048
Vaccinees	0.324	0.001

[a] Spearman's test was used for correlation analyses.
[b] AI, avidity index; MBC, memory B cell response.

for T cell dependent antigens has been shown previously for a number of important systemic infections, including dengue virus, rubella virus, and cytomegalovirus infections, as well as following vaccination for measles, *Haemophilus influenzae*, and pneumococcal disease (16, 17, 19, 21–23). T cell-independent antigens, such as LPS, activate antigen-specific B cells to proliferate and mature directly through Toll-like receptors (TLRs) or with the help of dendritic cells (34, 35). Dendritic cells utilize a nondegradative endocytic pathway to present T cell-independent antigens to B cells, and this leads to B cell proliferation, isotype switching, and maturation of high-affinity antibody production outside GC and without T cell help (34, 36–39). In the lamina propria, B cell activation leads predominantly to class switching to IgA2 (40). In Peyer's patches, class switching is similarly biased to the IgA isotype (41). Class-switched mature B cells then circulate, rehome to mucosal surfaces, and mature into plasma cells that produce IgA for secretion into the intestinal lumen. High-affinity, secretory IgA antibodies at mucosal surfaces neutralize microbial toxins and pathogens to help provide protection from disease with the help of high-affinity IgA antibodies (42).

The mechanism of protective immunity after cholera or cholera vaccination is currently not fully understood. Our previous studies suggest that protective immunity may be directed largely to the T cell-independent antigen LPS and that the development and particularly maintenance of memory B cells to LPS may differ strikingly between individuals infected with *V. cholerae* and those receiving a killed, oral cholera vaccine (25), potentially explaining the relatively short-term protection conferred by current cholera vaccination. In this study, we show that the antibody avidities of both IgA and IgG antibodies to CTB and to LPS correlate with memory B cell development to the same antigens, consistent with both events occurring as a result of B cell proliferation and maturation. We also show that the duration of high-affinity antibodies to both CTB and LPS is significantly different in patients and vaccinees, similar to the results with memory B cells.

Antibody avidity is known to be a reliable surrogate marker of immunological memory for systemic infections (22). Several studies have shown that both antibody avidity and the memory B cell response are indicators of previous exposure to a pathogen. The current protocol for measuring memory B cell responses that we have used in our studies (9, 10) is based on polyclonal stimulation of peripheral blood mononuclear cells (PBMCs) by mitogens for 6 days, followed by an ELISPOT procedure to estimate spot-forming ASCs; this technique is best carried out with fresh blood, may require substantial volumes of blood, and takes a week to complete. Measurement of antibody avidity by ELISA, on the other hand, is quicker, requires less blood, does not require mitogen stimulation, can be multiplexed for multiple antigens and multiple antibody isotypes, and can be done on stored serum or plasma samples historically collected. In this study, the correlation coefficients, although significant, were low, but our results suggest the potential use of the measurement of antibody avidity in plasma or serum as a potentially useful and less expensive alternative than measurement of memory B cell responses to predict protective immunity following infection or vaccination, particularly in facilities with limited resources and laboratory infrastructure.

In this study, the antibody avidities of two key antigens of CTB and LPS were measured in both patients and vaccinees to determine if a correlation of protection could be found. A vibriocidal antibody response is the current surrogate marker of protection against *V. cholerae* O1 infection, which is largely associated with anti-LPS antibody responses. Also, the level of serum IgA specific to LPS was found to be associated with protection against cholera (6). In this study, we showed that patients and vaccinees develop highly avid antibodies to both CTB and LPS, although the CTB-specific IgG or IgA antibodies with high affinity persisted for longer time period than those to LPS. It is possible that protective efficacy studies using measurements of antibody avidity to LPS may show some correlation in the future. This study was carried out in a country where cholera is endemic, Bangladesh, where infection with *V. cholerae* and enterotoxigenic *Escherichia coli* (ETEC) is prevalent. Individuals are preexposed to the different antigens and hence can have preexisting memory B cells. In this study, we have seen significant increases of CTB- and LPS-specific IgG and IgA antibody responses in vaccinated individuals on day 3 after the first dose of vaccine conferring preexisting memory B cells in the study participants.

In summary, our results show that both cholera patients and those receiving a killed, oral cholera vaccine develop highly avid antibodies of both the IgG and IgA isotypes to both the T cell-dependent antigen CTB and the T cell-independent antigen LPS and that the avidity indices of these antibody responses correlate well with measurement of memory B cell responses at corresponding time points, suggesting that both events may be occurring as part of B cell proliferation and maturation. Patients with cholera have a longer duration of highly avid antibodies and of memory B cells to both antigens, particularly to the T cell-independent antigen LPS, than do individuals receiving the cholera vaccine, consistent with the shorter duration of protection afforded by the vaccine. Improved understanding of all the events leading to B cell maturation to T cell-independent antigens in gut mucosa and the lamina propria may provide additional insights of relevance to improving the duration of protection obtained from current cholera and other mucosal vaccines. Antibody avidity may also provide a simpler and more widely available assay for measuring B cell proliferation and maturation events after infection and vaccination than the current memory B cell assay.

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