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Serum IgG Response to *Cryptosporidium* Immunodominant Antigen gp15 and Polymorphic Antigen gp40 in Children with Cryptosporidiosis in South India[▽]

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The surface-associated glycopeptides gp40, one of the most polymorphic *Cryptosporidium* antigens, and gp15, one of the most immunodominant *Cryptosporidium* antigens, are putative vaccine candidates because they mediate infection *in vitro* and induce immune responses *in vivo*. We evaluated antibody responses to these antigens before and after the first episode of symptomatic cryptosporidiosis in 51 children from a birth cohort study in an area in South India where *Cryptosporidium* is endemic and a major cause of parasitic diarrhea. IgG levels to gp15 and to homotypic and heterotypic gp40 antigens were measured in pre- and postdiarrheal sera by enzyme-linked immunosorbent assay (ELISA). There was a significant IgG response to gp15 ($P < 0.001$) following the first episode of cryptosporidial diarrhea. Using a general additive model, we determined the estimated time of the peak IgG response to gp15 to be 9.3 weeks (confidence interval, 5.2 to 13.4) following the diarrheal episode. In a subset of 30 children infected with *Cryptosporidium hominis* subtype Ia, there was a significant difference in IgG responses to homotypic *C. hominis* Ia and to heterotypic *Cryptosporidium parvum* II gp40 antigens ($P = 0.035$). However, there was also a significant correlation ($P = 0.001$) in the responses to both antigens in individual children, suggesting that while responses are in part subtype specific, there is significant cross-reactivity to both antigens. This is the first report of the characterization of immune responses to cryptosporidiosis in Indian children and the first study to investigate human immune responses to the polymorphic gp40 antigen. However, further studies are needed to determine whether immune responses to these antigens are protective against subsequent infections.

Cryptosporidium spp. are frequent causes of infectious diarrhea in children in developing countries (reviewed in references 19, 23, and 27). In these countries, malnourished children are at greater risk of acquiring cryptosporidiosis, and in turn, the disease is more severe in malnourished than in well-nourished children (reviewed in references 19 and 23). Early childhood cryptosporidiosis in these areas may lead to worsening malnutrition and growth faltering as well as to physical and cognitive deficits (15, 16, 19, 24, 34). Treatment options for cryptosporidiosis are limited. Nitazoxanide, the only drug that has shown some efficacy in immunocompetent individuals (48), is not effective in immunocompromised patients (1) and has not been widely evaluated in children, particularly those who are malnourished, in developing countries. There is no vaccine available for the prevention of cryptosporidiosis.

Among *Cryptosporidium* species, *C. parvum* and *C. hominis* cause most human infections, with *C. hominis* predominating in developing countries (reviewed in reference 54). Cryptosporidiosis in children is widespread in India (4, 8, 29, 32, 36, 37) and is the major cause of parasitic diarrhea in children under the age of 5 in South India (3, 4). The most common species identified in Indian children is *C. hominis* (3, 18, 36).

Immune responses to *Cryptosporidium* are poorly understood, and the correlates of protective immunity are not known. While cell-mediated immunity is crucial for resistance to and resolution of infection, antibodies may play a role in preventing the parasite from attaching to and invading host cells during its invasive stages (reviewed in references 44 and 12). In adults who are naturally or experimentally infected with *Cryptosporidium*, antibody responses are associated with partial protection from subsequent challenge, and the presence of preexisting antibodies is associated with decreased severity and duration of infection (reviewed in references 12 and 44). However, it remains to be determined whether these responses are themselves protective or whether they are reflective of protective cellular responses (44). Regardless, knowledge of immune responses to putative protective antigens is essential in order to design immune-based preventive strategies.

Recent efforts to identify putative protective antigens have focused on surface-associated proteins that mediate attachment to and invasion of host cells by invasive stages of the parasite (reviewed in references 12 and 51). The best studied of these antigens are gp40 and gp15, proteolytic cleavage products of a major surface glycoprotein, gp40/15 (also called GP60 or S60), which we (14) and others (43, 49, 52) have cloned and characterized. gp15 (also known as Cp17 or S16) is the C-ter-

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minal cleavage product of gp40/15 and is one of the most immunodominant *Cryptosporidium* antigens identified to date. The presence of preexisting anti-gp15 antibodies is associated with protection from diarrhea in naturally or experimentally infected adults (reviewed in references 12 and 44). gp15 has also been shown to induce gamma interferon-mediated cellular responses in previously infected humans (40).

gp40, the N-terminal cleavage product of gp40/15, is a secreted, mucin-like glycoprotein that associates on the parasite surface with the glycosylphosphatidylinositol (GPI)-anchored gp15 glycopeptide (39) and mediates attachment to and subsequent invasion of host cells (14, 51). The gene encoding gp40/15 is one of the most polymorphic genes identified in *Cryptosporidium* spp. (reviewed in references 54 and 28). This high degree of polymorphism in gp40 is consistent with the possibility that it is a virulence determinant that is under selective host immune pressure. Because of the extensive polymorphisms, the *gp40/15* locus is the most widely used for subtyping of clinical and environmental samples (54). Most of the polymorphisms are clustered in the hypervariable region of the gp40 part of the molecule, while gp15 is relatively conserved (31, 38, 50). Although gp40 has been shown to induce humoral and cell-mediated immune responses in mice (10, 47), it is not known whether this protein is immunogenic in infected humans or whether immune responses to it are species or subtype specific.

Previously, we investigated the clinical features and molecular and spatial epidemiology of cryptosporidiosis in a birth cohort of children in a semiurban slum community in South India (2, 3). The most common species identified in diarrheal stool samples from these children was *C. hominis*, with *gp40/15* subtype Ia predominating. The overall goal of the present study was to assess serum antibody responses to gp15 and gp40 after the first episode of cryptosporidial diarrhea in the same cohort of children and to determine if antibody responses to gp40 are species and subtype specific.

(These data were presented, in part, at the 44th Annual Meeting of the Infectious Diseases Society of America, Toronto, Canada, October 2006.)

MATERIALS AND METHODS

Study subjects and samples. Fifty-three children with 58 episodes of cryptosporidial diarrhea (defined as one or more episodes of diarrhea associated with the presence of *Cryptosporidium* spp. in the stool detected by microscopy) were enrolled in the study.

These children were part of a birth cohort of 452 children enrolled in a study on rotavirus infection (9, 22) and were monitored from birth until the age of 3 years. Briefly, the cohort was recruited from the semiurban slum areas of Ramnaickapalayam, Chinnallapuram, and Kaspa in Vellore, South India. An episode of diarrhea was defined as at least 1 day of diarrhea (three or more watery stools in a 24-h period) preceded and followed by 2 or more days without diarrhea. Stool samples were collected during all diarrheal episodes and fortnightly for surveillance. Serum was collected within 3 to 6 weeks of rotaviral diarrhea and at specific time points every 3 months during the first year and at 6-month intervals thereafter. Serum samples were stored in aliquots at −20°C prior to testing. The study was approved by the Institutional Review Board of Christian Medical College, and informed consent was obtained from the parents of the children enrolled in the study.

The average age (± the standard deviation [±SD]) of the children during their first episode of cryptosporidial diarrhea was 16.8 (±8.4) months. The clinical and sociodemographic characteristics of these children and the results of the molecular characterization of the *Cryptosporidium* spp. identified in their stools have been previously published (3).

Serum samples from 51 of the 53 children collected before (prediarrheal) and after (postdiarrheal) the first episodes of cryptosporidial diarrhea were available for testing in the current study.

ELISA. Sequences encoding gp40 and gp15 from *C. parvum* subtype II (GCH1 isolate), obtained from Saul Tzipori, Tufts Cummings School of Veterinary Medicine, North Grafton, MA, gp40 from *C. hominis* subtype Ia (TU502 isolate), also obtained from S. Tzipori, and a control protein containing the His, thioredoxin, and S-tags alone (14) were cloned into the pET32Xa/LIC vector (Novagen). The recombinant (r) proteins were overexpressed in *Escherichia coli* and purified by metal affinity chromatography as described previously (14, 40). Levels of serum IgG against gp15 and gp40 were quantified by enzyme-linked immunosorbent assay (ELISA) as previously described (30), using recombinant forms of *C. parvum* subtype II gp15 (rCpgp15 II) (5a) and gp40 (rCpgp40 II) and *C. hominis* subtype Ia (rChgp40 Ia) as antigens. Briefly, 96-well microtiter plates (Nunc, Rochester, NY) were coated with recombinant test or control proteins at a concentration of 0.4 μg protein/well. Excess antigen was washed off with 20 mM sodium phosphate–150 mM sodium chloride (pH 7.2; phosphate-buffered saline [PBS]) containing 0.05% Tween 20, and nonspecific binding was blocked with 1% bovine serum albumin (BSA) in PBS. Wells were then incubated with serum diluted 1:100 in PBS with 1% BSA for 1 h at 37°C. After being washed three times, wells were incubated with 50 μl of alkaline phosphatase-conjugated goat-anti-human γ chain-specific IgG (Sigma, St. Louis, MO) diluted 1:5,000 in 0.25% BSA/PBS. After being washed, wells were incubated with 50 μl of substrate solution (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) containing *p*-nitrophenyl phosphate (1 mg/ml; Sigma, St. Louis, MO) at room temperature. The reaction stopped after 15 min, and absorbance was read at 405 nm (A_{405}). The same positive- and negative-control sera (sera that were positive or negative by ELISA and Western blotting using *C. parvum* lysate as the antigen) were run on each plate to control for plate-to-plate variation. All samples were run in triplicate, and the mean A_{405} was determined. A_{405} values for the control protein containing only the fusion tags were subtracted from the A_{405} values for the patient sample. To adjust for interplate variability, values were normalized by dividing the A_{405} value of the sample by the A_{405} value of the positive control for that plate and multiplying by 100. The results were expressed as ELISA units (EU) (30).

Statistical analysis. Data were analyzed using Stata 10.1 for Windows (StataCorp, College Station, TX) and R 2.10.0 statistical software (http://www.r-project.org/). The differences in serum IgG levels pre- and postdiarrhea were compared using the paired *t* test, and log-fold changes in IgG levels postdiarrhea were compared to a null hypothesis of no change using the one-sample *t* test. The correlation between pre- and postdiarrheal serum IgG levels was calculated using Spearman's rank-order correlation coefficient.

Generalized additive model for analysis of serological response. Assuming that the serum IgG response following a diarrheal episode was nonlinear and time dependent, we aligned pre- and postdiarrheal levels of anti-gp15 IgG with respect to the time of the first diarrheal episode (considering it to be at time 0). In order to obtain the timing of the peak IgG response to gp15, curve fitting was performed using the generalized additive model (53; R. Sarkar, submitted for publication) with cubic splines supported by 5 knots. The quality of the fit was determined based on adjusted R^2 values. Modeling was performed with and without one pair of IgG EU values that had an unusual (reverse) pattern and resulted in a predicted curve and its confidence interval (CI). The uncertainty boundaries for the timing of the peak serum IgG response were then obtained by simulating the curve for the period of 4 to 20 weeks postdiarrhea, with a refined time increment of 0.01 week.

RESULTS

IgG response to gp15 before and after the first episode of cryptosporidial diarrhea. Serum anti-gp15 IgG levels assessed in 51 children before and after the first episode of cryptosporidial diarrhea showed a significant increase from the mean (±SD) prediarrheal level of 51.5 (±78.5) EU to the mean postdiarrheal level of 171.9 (±128.6) EU ($P < 0.001$) (Fig. 1A). In these children, there was a mean 1.5 (±1.3)-log-fold increase in serum IgG levels after a diarrheal episode ($P < 0.001$) (Fig. 1C). There was no significant correlation between pre- and postdiarrheal IgG levels (Spearman's $\rho = 0.154$; $P = 0.281$) for each child (Fig. 1B), indicating that the response to the infection did not depend on the baseline values.

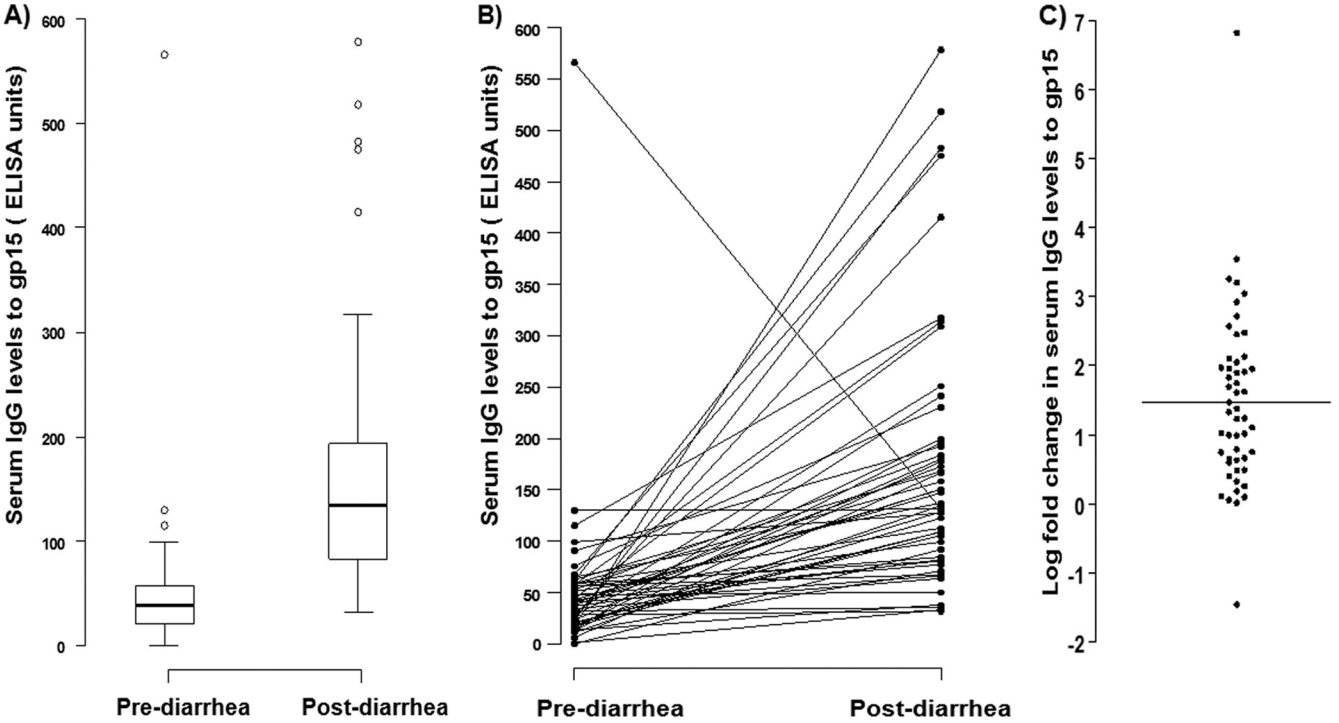

FIG. 1. Serum IgG responses to gp15 in children following the first episode of cryptosporidial diarrhea ($n = 51$). (A) Box plots of serum IgG levels in pre- and postdiarrheal samples. (B) Pre- and postdiarrhea levels of IgG in response to gp15 in each child. (C) Log-fold increase in serum IgG levels following the first episode of diarrhea.

Time to peak serum IgG response to gp15. When we plotted the data with respect to episode timing (Fig. 2A), we observed that the time to the episode and the time since the episode varied, and increases in EU values were unevenly distributed with respect to the postepisode sample's timing. Analysis of the timing of serum sample collection revealed that sera were collected at a mean (±SD) of 8.6 (±6.9) weeks for prediarrheal sera and a mean of 8.6 (±5.2) weeks for postdiarrheal sera, indicating that the intervals between the diarrheal episode and both pre- and postsample collection were comparable ($P = 0.985$) (Fig. 2A). When the generalized additive model was applied to these data (Fig. 2B), the mean (confidence interval) predicted difference in anti-gp15 IgG levels between the pre- and postdiarrheal sera was 60.2 (±10.7) EU and the predicted maximum difference in anti-gp15 IgG levels between the pre- and postdiarrheal sera was 138.1 (±18.5) EU. The model predicted the time of peak antibody response to be 9.3 weeks (CI, 5.2 to 13.4) following a diarrheal episode. When the model was run without a pair of sera (outlier) with a reverse pattern, the results were not substantially affected (Fig. 2C). Here, the model predicted the time of peak antibody response to be 11 weeks (CI, 5.2 to 17.2). The predicted difference in anti-gp15 IgG levels between the pre- and postdiarrheal sera was 65.8 (±9.4) EU, and the predicted maximum difference in anti-gp15 IgG levels between the pre- and postdiarrheal sera was 148.5 (±16.8) EU.

IgG response to gp40 before and after the first episode of diarrhea in *C. hominis* subtype Ia-infected children. In our previous study on the molecular characterization of *Cryptosporidium* species and subtypes infecting the children in the cohort, we found that most children were infected with *C. hominis* and that the predominant *gp40/15* subtype was Ia (3). In order to determine if antibody responses to the polymorphic gp40 antigen were subtype specific or cross-reactive between antigens, we compared IgG levels in response to the homotypic gp40 antigen from the infecting species and subtype (*C. hominis* gp40 Ia) with those in response to the next-most-common heterotypic gp40 antigen (*C. parvum* gp40 IIa) in all 30 children who were infected with *C. hominis* subtype Ia. Comparison of pre- and postdiarrheal IgG levels for each child showed that there was a significant correlation between IgG levels before and after diarrhea in response to the homotypic antigen (Spearman's $\rho = 0.568$; $P < 0.001$) as well as to the heterotypic antigen (Spearman's $\rho = 0.678$; $P < 0.001$) (Fig. 3A and B). While there was a 0.6 (±1.6)-log-fold increase in mean (±SD) IgG levels in response to the homotypic antigen following the episode of diarrhea, there was a 0.4 (±2.7)-log decrease in mean (±SD) IgG levels in response to the heterotypic antigen, and this difference in response was statistically significant ($P = 0.035$) (Fig. 3C and D). However, comparison of the log-fold change in IgG levels for each individual child showed that there was a significant correlation in the levels of antibody against both (Spearman's $\rho = 0.598$; $P < 0.001$) (Fig. 3E). These results suggest that while serum IgG responses to gp40 are in part subtype specific, there is cross-reactivity between homotypic and heterotypic antigens.

The postdiarrheal levels of IgG in response to *C. hominis* gp40 Ia and *C. parvum* gp40 II in children infected with *C. hominis* subtype Ia showed greater individual variability than those in response to the gp15 antigen. This finding, along with

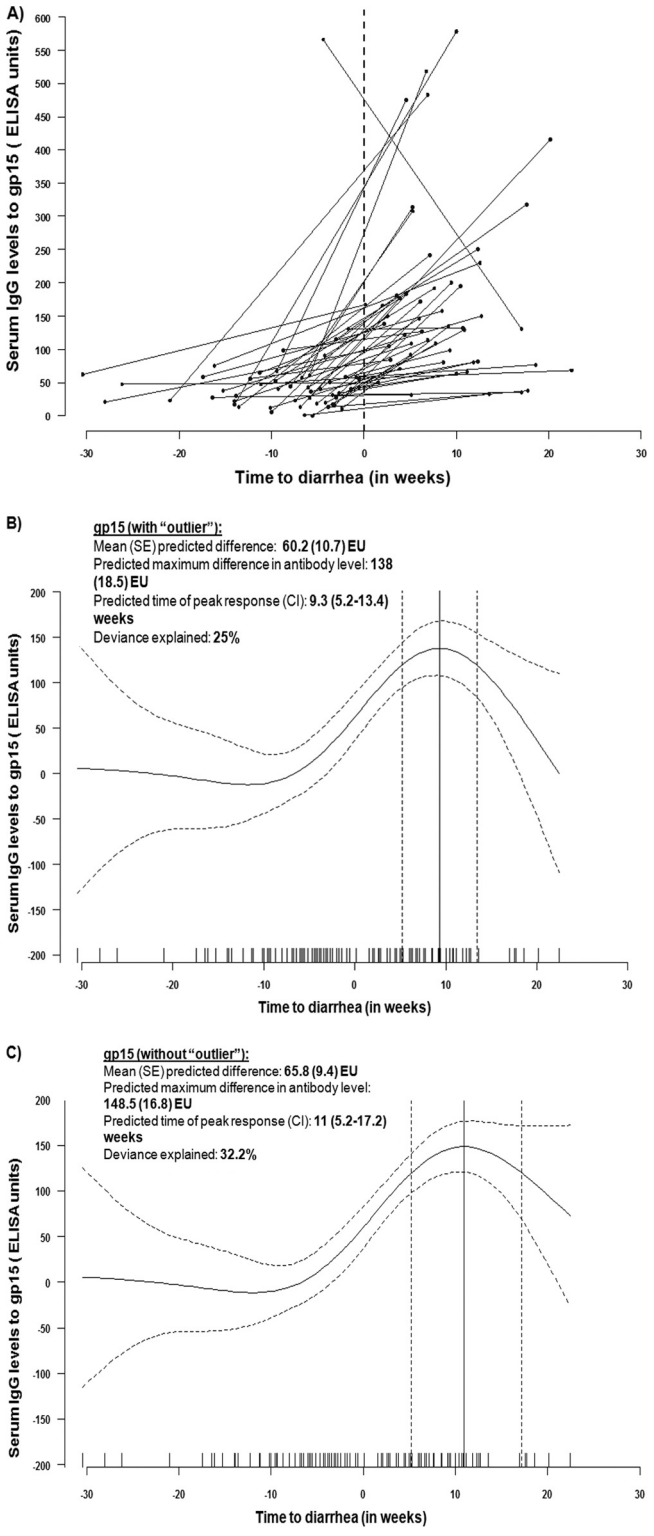

FIG. 2. Generalized additive model to estimate time to peak serum IgG levels in response to gp15 following the first episode of cryptosporidial diarrhea ($n = 51$). (A) Times of pre- and postdiarrhea serum collection in relation to first diarrheal episode (week 0). The dashed line indicates week 0. (B and C) Generalized additive model demonstrating the peak antibody response in the weeks following the diarrheal episode with the outlier (B) and without the outlier (C). The dashed vertical lines indicate the lower and upper confidence interval for the predicted time of peak response; the solid vertical line indicates the predicted time of peak responses; the dashed plot lines indicate the lower and upper confidence interval for the predicted maximum difference in antibody level; the solid plot line indicates the predicted maximum difference in antibody level.

the fact that there were fewer data points (51 children in the study versus 30 children infected with *C. hominis* subtype Ia), resulted in wider confidence intervals for the time of peak response as determined by the general additive model (data not shown), so no meaningful conclusions about the timing of the response could be drawn.

DISCUSSION

Although *Cryptosporidium* spp. are well known to be a major cause of parasitic diarrhea in children and HIV-infected adults in India (5, 6, 20, 45), this is the first study to investigate immune responses to this parasite in India. In our study, the birth cohort design and the collection of stool samples from all diarrheal episodes enabled us to identify the first episode of symptomatic cryptosporidiosis in children in the cohort and to investigate the immune responses to putative protective antigens resulting from this episode. We found a strong antibody response to the immunodominant gp15 antigen (35, 42), which was predicted to peak ~9 weeks after the episode, and showed for the first time that the polymorphic gp40 antigen induces humoral immune responses in infected humans and that these responses, while in part subtype specific, are cross-reactive between homotypic and heterotypic antigens.

Previous studies have documented an immune response to gp15 in symptomatic infections in adults and children (33, 42, 46) (Allison et al., submitted) and in urban populations following waterborne outbreaks of cryptosporidiosis (21, 33). The data from our study in a semiurban slum in a developing country where cryptosporidiosis is endemic confirms and extends these findings. We found a highly significant serum IgG response to this immunodominant antigen in children following the first episode of cryptosporidial diarrhea. In our study, although most children were infected with *C. hominis*, we used recombinant gp15 derived from *C. parvum* as the antigen in the ELISAs, since gp15 is relatively conserved between the two species. In a previous study on antibody responses to gp15 in Bangladeshi children with diarrhea, we found a highly significant correlation between serum antibody responses to recombinant gp15 derived from both species even though most children were infected with *C. hominis* (Allison et al., submitted). Preidis et al. found that *C. hominis* but not *C. parvum* gp15 induced gamma interferon-mediated cellular immune responses in adult volunteers with serological evidence of prior *Cryptosporidium* infections (40). However, the infecting *Cryptosporidium* species in their study was not known.

In a novel approach, we applied the generalized additive model to these data and calculated the predicted time to the peak antibody response after an episode of diarrhea. Other investigators have used this temporal modeling approach to study the impact of vaccination on the incidence of hepatitis B in infants (25), the emergence of fluoroquinolone resistance (11), the time of HIV infection in hemophiliacs (7), etc., but to

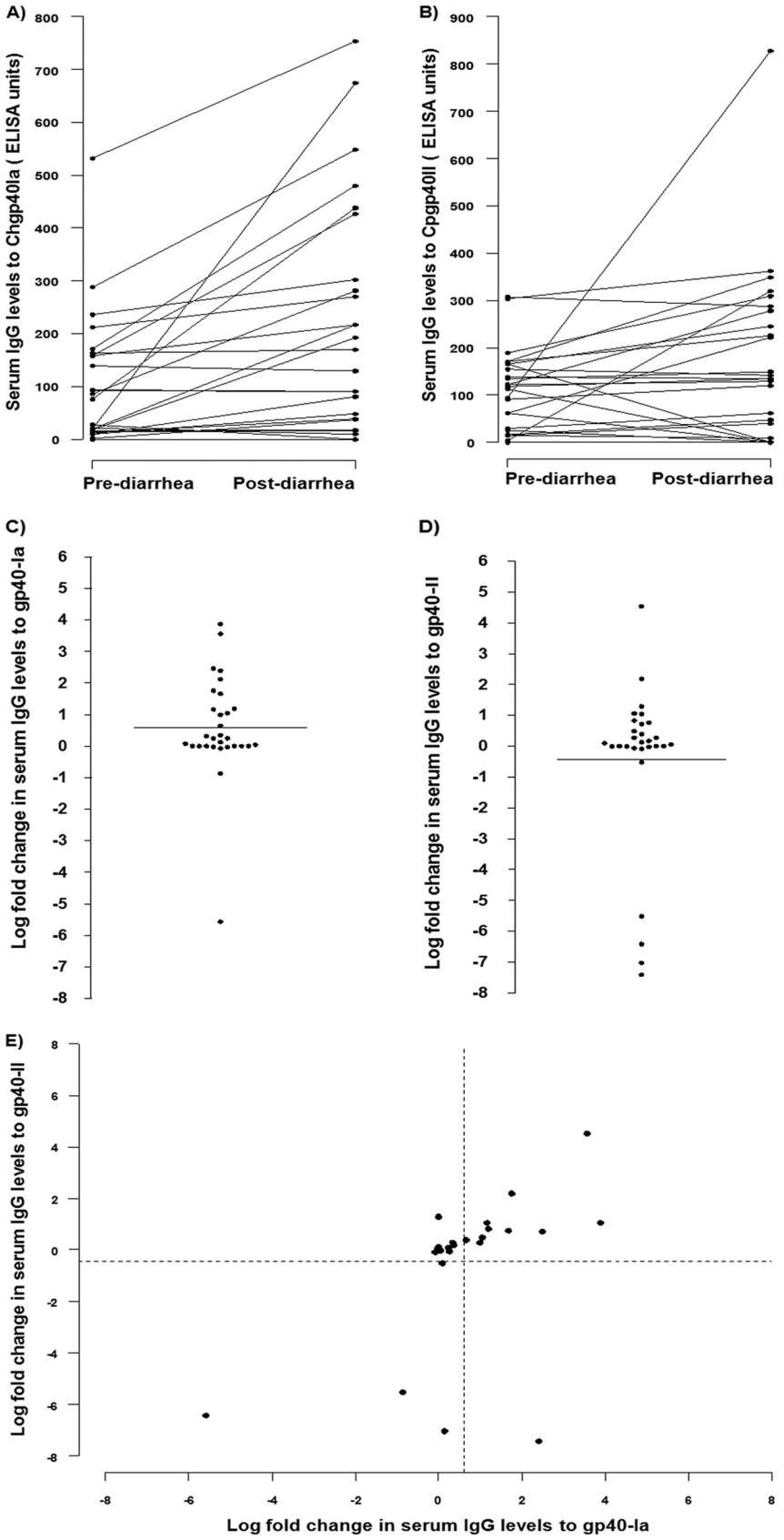

FIG. 3. Serum IgG responses to homotypic and heterotypic gp40 in children infected with *C. hominis* subtype Ia (n = 30). (A and B) Pre- and postdiarrhea serum IgG levels in response to Chgp40Ia (A) and Cpgp40II (B) in each child. (C and D) Log-fold increase in IgG levels in response to Chgp40Ia (C) and Cpgp40II (D). (E) Correlation of log-fold changes in serum IgG levels in response to Chgp40Ia and Cpgp40II IgG in each child infected with *C. hominis* subtype Ia. The dashed vertical line and the dashed horizontal line indicate the mean log fold change in serum IgG levels to Chgp40 Ia and II, respectively.

our knowledge this is the first time it has been applied to data to model antibody responses over time. Validating this statistical approach, the predicted prediarrheal anti-gp15 IgG levels and the mean rise in IgG levels were similar to the mean IgG level measured by ELISA. Application of these statistical tools to serological data can provide an approach to understanding the dynamics of humoral immune responses over time. In other studies, seroconversion following an episode of diarrhea has been used as a more sensitive marker of infection than direct detection of a *Cryptosporidium* sp. in stool samples (41) and as a method to evaluate interventional tools to improve water quality (17).

The predominance of a single species (*C. hominis*) and *gp40/15* subtype (Ia) in the study area (3) facilitated a natural experiment on species- and subtype-specific responses to the polymorphic gp40 antigen from both major *Cryptosporidium* species in these children. The mucin-like gp40 antigen mediates infection *in vitro* (13) and may thus serve as a target for preventive or interventional modalities. This antigen contains both conserved and polymorphic domains. The N-terminal polyserine domain is conserved (except for the number of serine residues) (14, 49, 52) among all species and subtypes identified thus far and is predicted to be heavily O glycosylated (H. Ward, unpublished data). The region that is C terminal to the polyserine domain is highly polymorphic among all known species and subtypes and contains only a single conserved serine residue (Ward, unpublished). If gp40 is to be considered a putative vaccine candidate, it is essential to determine whether immune responses to this antigen are species and subtype specific or cross-reactive among different polymorphic forms and whether these responses are directed at glycan, peptide, or glycopeptide epitopes.

The findings of our study indicate that antibody responses to nonglycosylated peptide epitopes of gp40 do occur, since the antigens used for ELISA were *E. coli*-derived recombinant proteins that are not glycosylated. The significant fold increase in serum IgG levels in response to both heterotypic and homotypic gp40 antigens and the significant correlation between responses to both antigens following the first episode of symptomatic cryptosporidiosis in these children suggest that there is cross-reactivity between the antigens, most likely to peptide epitopes in the conserved polyserine domain. However, the finding that the fold increase in IgG levels was significantly greater in response to the homotypic *C. hominis* subtype Ia gp40 antigen than to the heterotypic *C. parvum* subtype IIa antigen in children infected with *C. hominis* subtype Ia suggests that subtype-specific responses may also occur, most likely to peptide epitopes in the hypervariable domain.

In conclusion, we took advantage of samples and data from an existing birth cohort study and incorporated molecular, immunological, and statistical techniques to study antibody responses to the immunodominant gp15 and polymorphic gp40 antigens following the first episode of symptomatic cryptosporidiosis among children in a developing country where *Cryptosporidium* spp. are endemic. Both gp15 and gp40 are surface-associated glycopeptides that are actively involved in the process of sporozoite attachment to and invasion of host cells and that induce antibody and cell-mediated immune responses and are therefore attractive vaccine candidates (10, 26, 51). This study is an initial step in determining whether either or both of these antigens could be targeted for vaccine development.

However, there are a number of limitations to our study. In addition to the small number of subjects, we were limited to investigation of systemic antibody responses to peptide epitopes of gp15 and gp40 following the first episode of symptomatic cryptosporidiosis. Since this retrospective study was designed for analysis of the correlates of immunity to rotavirus infection, there was no screening for asymptomatic cryptosporidial infections, and therefore the sera were collected at various time points in relation to the diarrheal episode associated with cryptosporidiosis and so could not be temporally correlated to a specific episode of cryptosporidial infection. In addition, we were not able to investigate cell-mediated responses or to determine whether humoral or cellular immune responses are directed at glycan or glycopeptide epitopes of both antigens or at conserved or polymorphic domains of gp40 and, most importantly, whether immune responses to either or both of these antigens induce protective immunity. Currently, our efforts are directed at investigating these possibilities in a prospective, longitudinal study of systemic and mucosal cellular and humoral immune responses to peptide and glycopeptide forms of these antigens expressed in *Toxoplasma gondii* (39) during symptomatic and asymptomatic cryptosporidiosis in a birth cohort of children in this community.

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