Specific Immunoglobulin A Antibodies in Maternal Milk and Delayed *Helicobacter pylori* Colonization in Gambian Infants

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Background. Immunoglobulin A (IgA) in maternal milk may protect Gambian infants from early *Helicobacter pylori* colonization. This study sought evidence that this protection could be due to specific IgA antibodies.

Methods. Sixty-five infants were screened from 12 weeks of age with [13C]-urea breath tests. Antibodies in maternal milk were measured to determine total IgA content and to detect specific IgA antibodies against crude whole-cell and recombinant *H. pylori* urease antigen preparations.

Results. Ten children (15%) had no evidence of early *H. pylori* colonization, 10 (15%) had early *H. pylori* colonization, and 43 (66%) had mixed results. Levels of maternal circulating specific immunoglobulin G, total milk IgA, and IgA directed against crude whole-cell *H. pylori* antigen preparation were not significantly associated with the rate of infant *H. pylori* colonization. However, mothers of infants with no evidence of early colonization produced significantly higher levels of anti–recombinant urease IgA antibodies in milk than did control mothers, particularly at 8, 16, and 20 weeks postpartum (*P* < .01).

Conclusions. These observations support the hypothesis that antibodies in mother’s milk directed against *H. pylori* urease can protect against colonization in human infancy.

Chronic *Helicobacter pylori* infection is the most common cause of upper gastrointestinal disease in humans [1]. Colonization often begins in childhood, particularly in developing nations [2], such as The Gambia [3, 4].

In humans, there is no evidence that the immune response either cures infection or protects against re-colonization after eradication of infection [5]. However, breast feeding may protect infants from early *H. pylori* colonization [6], although conflicting data have been produced from cross-sectional studies [7]. We have previously reported an association between delayed infant colonization with *H. pylori* and high levels of anti–*H. pylori* IgA antibodies in mother’s milk [8] in a country where prolonged breast feeding of infants is normal [9]. The data from this earlier report, although suggesting that specific IgA antibodies in milk play a role in protecting against infant colonization, did not consider the possible protective effect of total IgA levels in maternal milk, nor did they identify specific bacterial antigens that might be the target of protective antibodies. This study was therefore undertaken to determine whether protection against infant *H. pylori* colonization attributable to maternal milk was conferred by a specific effect or was secondary to high secretion of total IgA in individual mothers and to identify possible antigen targets of potentially protective milk antibodies.

**METHODS**

**Study population.** The study took place at the Medical Research Council (MRC) Research Station in Keneba, The Gambia, a village of rural subsistence farmers living in family compounds. All infants born over a 15-month period and their mothers were recruited for the study, subject to informed parental consent obtained.
by local fieldworkers. Study protocols were approved by the Joint MRC and Gambian Government Ethical Committee and by a meeting of village elders. Eighty-one infants were born from August 1993 through October 1994, 65 of whom consented to be included in the study. All village mothers breastfed their infants throughout the study period.

Children and their mothers were evaluated at ∼4-week intervals from 4 weeks to 1 year postpartum. On study days, mothers collected a 5 mL-sample of milk from each breast by manual expression before the first feed. From the age of 12 weeks, [13C]-urea breath tests were performed for the children on each study day. In addition, 0.5 mL-samples of capillary blood were collected once from each mother.

[13C]-urea breath test. The [13C]-urea breath test is a reliable, noninvasive means of diagnosing H. pylori colonization. Cut-off values used to distinguish positive from negative test results in adults cannot be directly applied to populations of young children for reasons such as differences in CO2 production rates [10]. We therefore used a protocol and cut-off that have been endoscopically validated among Gambian children aged <2 years [11]. In brief, a baseline breath sample was obtained from children shortly after awakening. Immediately thereafter, a drink containing 50 mg of [13C]-urea (99% atom excess; Cambridge Isotopes), 50 mg of naturally abundant urea, and 0.5 mg/kg of lactulose elixir BP, and a test meal of 5 g of glucose polymer (Polycose; Abbott Laboratories) dissolved in an appropriate amount of water were given. Lactulose elixir was given so that concomitant measurement of breath hydrogen levels could be used to assess whether the substrate had reached a fermentative (and therefore potentially ureolytic) bacterial population in the large bowel or the contaminated small bowel before the collection of the second breath specimen 30 min later. Breath samples were transported to the MRC Human Nutrition Research laboratory (Cambridge, United Kingdom) and analyzed by gas isotope-ratio mass spectroscopy (SIRA 10; VG Isotech). Isotopic enrichment was expressed as ∆‰ relative to the international standard Pee Dee Belemnitelimestone (PDB).

The baseline corrected [13C] enrichment was defined as the difference in isotopic enrichment between the baseline breath sample and a breath sample obtained 30 min thereafter. To distinguish between positive and negative results of 5.47 ∆‰ relative to PDB, an appropriate cut-off baseline enrichment value was calculated as described elsewhere [11] and validated. The breath test was used to divide children into 3 categories. Children with negative breath test results at 12, 16, 20, and 24 weeks of age were classified as showing no evidence of early H. pylori colonization. Children with sequential positive breath test results at these ages were classified as showing evidence of early H. pylori colonization, and the remaining children were classified as showing mixed results.

Milk IgA ELISA. Three separate immunoassays were performed with milk samples to measure total IgA levels, specific IgA levels directed against whole-cell H. pylori antigen preparation, and specific IgA levels directed against a recombinant H. pylori urease antigen preparation. Total IgA levels in milk samples were measured as described elsewhere [12]. For specific immunoassays, a crude whole-cell H. pylori antigen was prepared [13] from a pool of 3 strains of H. pylori isolated from gastric biopsies obtained from Gambian adults. In brief, strains were grown on 10% blood agar (Thayer-Martin agar base; Merck) for 48 h in a microaerobic atmosphere and were harvested into phosphate buffered saline. Cells were lysed by sonication, and debris was removed by centrifugation at 7000 g for 10 min. The supernatant was recovered and used as antigen at a final soluble protein concentration of 6.25 ng/mL. An additional immunoassay was undertaken using recombinant urease (rUrease) antigen preparation at a final soluble protein concentration of 20 ng/mL. Recombinant urease was expressed in Escherichia coli and was purified as described elsewhere [14]. It contained both of the major urease subunits that have been shown to assemble into the native structure but was devoid of enzyme activity.

Milk samples were frozen within 1 h after collection, were stored at −20°C, and were transported, frozen, to the United Kingdom for analysis. Prior to performing the assay, the aqueous phase was separated from cellular debris and the fatty layer. For the 2 specific IgA immunoassays, this aqueous phase was then absorbed at 4°C overnight, against monoclonal antibodies to Lewis b antigen (Alpha Laboratories) and crude Campylobacter jejuni antigen [13]. Absorbed milk samples were used in the assays at a final concentration of 1:200.

Bound milk IgA was identified using peroxidase-conjugated anti-IgA (α chain specific; Sigma), with ortho-phenylene diamine as indicator. Each assay plate contained 6 control milk specimens (chosen to cover the useful range of the assay), a reagent control, and 2 additional controls of mouse ascites—one from mice immunized against rUrease and the other from mice immunized against a non–urease-producing deletion-mutant strain of H. pylori [15]. Bound murine antibodies from these 2 controls were identified with peroxidase-conjugated anti-mouse immunoglobulin (Sigma) followed by addition of substrate and indicator. All samples and controls were assayed in duplicate. Provided that murine and reagent controls were acceptable, results were initially expressed as optical density ratios, corrected by reference to the mean values obtained from prior assaying of the 6 control milk samples. Optical density ratios were converted into relative concentrations by means of calibration curves and were then used to produce a proportional concentration of specific IgA by correcting for the total IgA concentration measured in each milk sample. As the relative concentrations were not quantified, the precise contribution of
H. pylori–specific IgA to total milk IgA was not determined, but simple titrations to end point suggest that H. pylori–specific antibodies comprised <1% of the total IgA reactivity in any individual sample. Assaying of specific serum IgG levels in mothers was performed by ELISA as described elsewhere [13], with use of the 2 specific antigen preparations separately.

**Western immunoblots.** Milk samples obtained from 10 mothers (selected according to ELISA results) were blotted against rUrease and crude whole-cell antigen preparations and against a crude whole-cell antigen preparation from a non–urease-producing deletion-mutant strain of H. pylori [15]. SDS-PAGE gels and nitrocellulose strips were prepared as described elsewhere [13]. Strips were incubated with test milk samples diluted 1:200 in Tris-HCl with 5% bovine milk protein. Bound milk IgA antibody was detected using alkaline phosphatase-conjugated goat anti-human IgA (Sigma), followed by 5-bromo-4-chloro-3-indoyl-phosphate substrate with nitroblue tetrazolium. The dried strips were then scanned using a flat bed scanner, and the data were calibrated against molecular weight standards and processed using Band LeaderTM (Technology) and Excel (Microsoft) software packages.

**Statistics.** More than 90% of possible milk samples were collected from mothers at approximately 4, 8, 12, 16, 24, 36 and 52 weeks postpartum and analyzed. A less complete data set was obtained from samples collected and analyzed at other time points, for a variety of logistical reasons. Further analysis and presentation of results was therefore restricted to samples obtained at 4, 8, 12, 16, 24, 36 and 52 weeks postpartum. Comparison of maternal milk antibody levels throughout lactation according to the child’s breath test results were made with Student’s unpaired t test (2-tailed).

**RESULTS**

Sixty-four of 65 mothers were IgG-seropositive, as revealed by analysis of crude whole-cell H. pylori antigen preparations (median optical density, 0.971; interquartile range, 0.69–1.15). The mothers also expressed IgG reactivities against rUrease, with optical densities ranging from 0.31 to 1.69 (median optical density, 0.96; interquartile range, 0.67–1.16). There was no correlation between the level of serum IgG response detected against whole-cell and rUrease antigen preparations in individual mothers.

Ten children (15%) had sequential negative breath test results up to 6 months of age and were classified as showing no evidence of early H. pylori colonization. Four of these children had a single missing data point and could have therefore been misclassified. Ten children (15%) had sequential positive breath test results to age 6 months and were classified as having early H. pylori colonization. Forty-three children (66%) had both positive and negative breath test results during the first 6 months of life and were classified for analysis as having mixed results. Two children had ≥2 missing data points and were therefore not included in the analysis.

There were no significant differences in maternal serum IgG optical density ratios against either of the H. pylori antigen preparations between mothers of children without early H. pylori colonization (mean whole-cell–antigen ratio, 0.404; 95% CI, 0.208–0.6), mothers of children with early H. pylori colonization (mean whole-cell–antigen ratio, 0.386; 95% CI, 0.175–0.597), and mothers of children with mixed test results (mean whole-cell–antigen ratio, 0.494; 95% CI, 0.36–0.628). Mean concentrations of total IgA in milk samples obtained 4 weeks postpartum were 1.34 mg/mL (95% CI, 1.09–1.6) among mothers of children without early H. pylori colonization and were not significantly different from concentrations among mothers of children with early H. pylori colonization (mean concentration, 1.53 mg/mL; 95% CI, 1.01–2.05) or among mothers of children with mixed test results (mean concentration, 1.5 mg/mL; 95% CI, 1.39–1.61). Concentrations of total IgA in milk gradually decreased throughout lactation [12], but no significant differences could be detected between mothers of children with or mothers of children without early H. pylori colonization at any stage of the study. The mean proportional concentration of IgA directed against crude whole-cell antigen preparations throughout lactation was also similar for mothers of children with early H. pylori colonization (mean proportional concentration, 0.66; 95% CI, 0.61–0.74), compared with mothers of children with early H. pylori colonization (mean proportional concentration, 0.68; 95% CI, 0.64–0.68) and mothers of children with mixed test results (mean proportional concentration, 0.72; 95% CI, 0.68–0.77).

The mothers of children with no evidence of early H. pylori colonization, however, produced significantly higher proportional concentrations of anti-rUrease IgA in their milk than did other mothers during the first 6 months (analysis of variance, P < .05 for milk samples obtained at 8, 16, and 20 weeks) (figure 1). Conversely, by 36 weeks, proportional concentrations of anti-rUrease IgA were greatest among mothers of children with early positive breath test results and were least among the mothers of children with negative test results. This is further illustrated by the cross-sectional data shown in figure 2, which demonstrate that anti-urease IgA levels started high but decreased throughout lactation among mothers of noncolonized children but started low and became elevated among mothers of children who developed H. pylori colonization, although these trends did not reach significance in this population.

Western blot tests, using crude whole-cell and rUrease antigen preparations, confirmed recognition of the major urease subunits by IgA in the milk of mothers of the 10 infants who showed no evidence of early H. pylori colonization. A typical Western blot result is shown in figure 3. The only protein band recognized in the rUrease preparation was the 67kDa subunit.
A band of 67 kDa was also only detectable on immunoblots using the crude whole-cell antigen preparation. Additional bands of milk IgA antibody recognizing other \textit{H. pylori} antigens were more clearly visualized on immunoblots that used an antigen preparation derived from the urease negative deletion mutant strain of \textit{H. pylori} than on those that used the crude whole-cell antigen preparation used in the ELISA.

**DISCUSSION**

This cohort study demonstrates that, in a population of infants at high risk of acquiring \textit{H. pylori} colonization and in which extended breast feeding is normal, the presence of specific human milk anti-rUrease IgA antibodies was associated with protection against \textit{H. pylori} colonization. \textit{H. pylori} is common in The Gambia, where most adults and children who have undergone endoscopic examination have been shown to be colonized [3, 11]. Virtually all Gambian adults are IgG seropositive, and colonization begins in early life [3, 4]. Our data suggest that a high level of exposure to infective \textit{H. pylori} is typical in this community. The source of infection is likely to be other colonized individuals, either by an oral/oral [16] or fecal/oral route [17]. An unexpected feature of longitudinal studies of young children from developing countries, first reported by Klein et al. [18], is the observation that positive urea breath test results are frequently followed by negative test results in many children. This has been observed in The Gambia [4] and elsewhere [19–21] and suggests that initial \textit{H. pylori} colonization in human childhood may be transient, as it is in some primate models [22]. An alternative explanation proposed by some investigators is that the urea breath test may be unduly sensitive (and therefore relatively nonspecific) in very young children [23]. We have overcome this problem by using sequential negative test results to identify children with no evidence of early \textit{H. pylori} colonization.

We have previously shown that levels of circulating specific IgG antibodies against \textit{H. pylori} antigens are slow to appear during natural colonization in human infancy [4]. Most systemic anti–\textit{H. pylori} IgG in children aged <6 months is likely to be maternally derived and transferred to the fetus via the placenta. IgG antibodies contribute to immunity against gastric infection with \textit{Helicobacter} species in some murine models [24], raising the possibility that maternal specific IgG may confer passive protection against \textit{H. pylori} in human infancy. However, we were unable to detect any association between maternal levels of circulating specific IgG antibody against crude whole-cell or rUrease antigen preparations and infant urea breath test results.

No differences in total IgA levels in milk or specific IgA directed against crude whole-cell \textit{H. pylori} antigen preparation were detected between mothers of children with or mothers of children without evidence of early \textit{H. pylori} colonization. This contrasts with data from our preliminary study, in which we used an antigen prepared by elution of soluble \textit{H. pylori} proteins and reported a protective effect of maternal milk antibodies against a crude whole-cell \textit{H. pylori} antigen [8]. It is likely that the eluate antigen used in that study contained proportionally more urease enzyme (and bacterial debris containing antigen) than the sonicated and centrifuged crude antigen preparation used in the present series.
H. pylori be more effective at inhibiting growth in vitro [25]. Human milk also inhibits the binding of H. pylori to epithelial cell surfaces in a manner independent of antibody [26], an effect which has also observed in milk samples from Gambian mothers [27]. Protection against colonization conferred by human milk is not absolute, as 85% of the breast-fed infants in this study had ≥1 positive breath test result, despite ingesting large quantities of milk and total IgA antibody [12]. This raises the possibility that the milk from mothers of children with no evidence of early H. pylori colonization may be more effective at inhibiting H. pylori than the milk of other mothers.

Our most exciting finding was the association between delayed infant colonization by H. pylori and elevated levels of specific IgA antibodies directed against rUrease in maternal milk. Mothers of children with no evidence of early H. pylori colonization showed significantly elevated levels of anti-rUrease IgA antibody in milk, compared with the other mothers, at 8, 16, and 20 weeks postpartum. This suggests that levels of specific antibody in maternal milk influence subsequent infant colonization. There is evidence that similar antibodies can lead to immunity in animal models of H. pylori infection [28]. Several research groups have successfully orally immunized mice against infection with Helicobacter species with urease preparations [29–33], and some have succeeded in the same model with other antigens [32]. This therapeutic immunization may be antibody independent [34], although Czinn et al. [30] in 1993 and Blanchard et al. [35] in 1995 have shown that an IgA monoclonal antibody against urease may prevent the ability of Helicobacter felis to infect mice.

From 16 weeks of age, there was a gradual decrease in specific antibody levels in milk among the mothers of noncolonized children and an increase in specific antibody levels in milk among mothers of children with positive breath test results. It is possible that this increase in specific antibody levels was caused by increased exposure of primed mothers to antigen secondary to their infants becoming colonized (figure 2).

In The Gambia, specific antibodies in milk directed against known bacterial colonization factors, such as urease, appear to protect against H. pylori colonization in infants. Whether this confers an advantage to children has yet to be established. There are 2 main implications of our work. First, the identification of naturally occurring human IgA antibodies that provide passive protection against H. pylori colonization supports the idea that active immunity against H. pylori colonization could be achieved with use of vaccines that stimulate production of the same class of antibodies. Second, because colonization with H. pylori occurs early in infancy in The Gambia, vaccine intervention strategies, should they be contemplated, will need to be applied soon after birth.

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References

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