Innate Immune Factors in Mothers' Breast Milk and Their Lack of Association With Rotavirus Vaccine Immunogenicity in Nicaraguan Infants

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To better understand underlying causes of lower rotavirus vaccine effectiveness in low-middle income countries (LMICs), we measured innate antiviral factors in Nicaraguan mothers' milk and immune response to the first dose of the pentavalent rotavirus vaccine in corresponding infants. No relationship was found between concentrations of innate factors and rotavirus vaccine response.

Keywords: breast milk; innate immunity; lactadherin; lactoferrin; Nicaragua; rotavirus vaccines.

Although the greatest burden of rotavirus disease is in LMICs, oral rotavirus vaccines (RVs) have lower efficacy in these settings [1–3]. In Nicaragua, the pentavalent RV (RV5) provides approximately 46% effectiveness against hospitalization for rotavirus diarrhea, compared with estimates of 82% to 95% in the United States [4, 5]. One possible explanation for lower effectiveness in LMIC infants is direct inhibition of this oral vaccine by acquired or innate immune factors present in breast milk. Breast milk inhibits RVs in vitro, activity that could be mediated by innate antimicrobial proteins. The innate factor lactoferrin is present in breast milk, and it inhibits rotavirus activity [6]. Another innate factor in breast milk, lactadherin, is associated with protection against symptomatic rotavirus infection [7]. Tenascin-C (TNC) is an innate, highly charged, hexameric protein present in breast milk that inhibits human immunodeficiency virus replication via interruption of virus-host receptor interactions [8], and it may also be important in the inhibition of other viral pathogens. These innate factors, together with acquired immunity in breast milk, could reduce virus replication in the infant gastrointestinal tract and blunt the infant's response to RVs. Furthermore, breast milk from LMIC mothers has higher concentrations of (1) lactoferrin and lactadherin and (2) rotavirus-specific immunoglobulin (Ig)A titers compared with mothers from high-income countries [9, 10], indicating a greater potential for RV inhibition. To address this concern, clinical trials of transient breastfeeding withholding have been conducted at the time of RV administration [11, 12]; in these trials, breastfeeding withholding for a total of 2 hours did not improve the infant's immune response to RVs.

We have previously shown that high titers of rotavirus-specific IgG antibody in maternal serum are associated with failure to seroconvert to RV5 in corresponding infants, whereas rotavirus-specific IgA titers in breast milk were not associated with seroconversion [13]. The objective of this study was to test whether there is an association between innate factors in breast milk and infants' RV-elicited immune responses. We examined the association between levels of lactoferrin, lactadherin, and TNC in breast milk, breast milk neutralization of RV5, and immunogenicity to the first dose of RV5 in respective infants. We hypothesized that high concentrations of innate factors in breast milk would be associated with decreased RV-elicited immune responses.

METHODS

Study Setting and Design

This study was performed in León, Nicaragua. At the time of the study, the national immunization schedule included RV5 at 2, 4, and 6 months of age. Mother-infant pairs were recruited in their households in September and October 2012, using public health rosters of pregnancies and live births in the Perla Maria and Subtaiva Health Sectors of León [13]. The recruitment visit occurred 1 day before the infant's first immunization visit. Eligibility criteria for infants included gestational age greater than 35 weeks at birth, birthweight of 2.5–4.5 kg, no known chronic health conditions, currently breastfeeding at least 4 times daily, eligible to receive RV5, and mother without known immune disorder or blood transfusion within

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the past 9 months. Informed consent was obtained from each mother for involvement of the mother-infant pair. The study was approved by institutional review boards of the Universidad Nacional Autónoma de Nicaragua, León and the University of North Carolina at Chapel Hill.

At recruitment, a preimmunization serum sample was obtained from each infant. At the same visit, breast milk and serum samples were obtained from each mother. The following day, infants received RV5 according to the national immunization schedule in the public health facility. A study nurse who accompanied each of the pairs to the infant's first immunization visit recorded breastfeeding patterns by direct observation. Four weeks after the first dose of RV5, a second serum sample was obtained from each infant in the household.

LABORATORY METHODS

Samples were transported at 4°C to the University Microbiology Laboratory where serum samples were separated and then stored at −70°C and breast milk samples were stored at −70°C. Commercial kits were used to assay breast milk concentrations of lactoferrin (Innovative Research Inc, Novi, MI) and lactadherin (Boster Bio, Pleasanton, CA). TNC concentrations in breast milk were determined by enzyme-linked immunosorbent assay (ELISA). In serially diluted samples, TNC was detected with mouse anti-TNC monoclonal antibody (Fisher Scientific) and antimouse peroxidase-conjugated antibody (Promega). Tetramethylbenzidine substrate was added and absorbance read at 450 nm. TNC concentration was determined by comparison to dilutions of quantitated purified TNC protein (Millipore, Billerica, MA), with detection limit of 4.88 ng/mL. Rotavirus-specific IgA titers in milk were assayed by enzyme immunoassay, and neutralizing antibody titers in milk were assayed using a microneutralization assay, both as previously described [9]. Serum rotavirus-specific IgA was assayed by ELISA [9, 14].

Statistical Analysis

Seroconversion to the first RV5 dose was defined as a 4-fold or greater increase in rotavirus-specific IgA titers in the infant's postimmunization serum sample compared with the preimmunization sample.Spearman's rank correlation coefficients (ρ) were estimated to examine correlations between concentrations of innate factors, between innate factors and breast milk antibody titers, and between innate factors and fold change in infant rotavirus-specific IgA titers before and after immunization. Wilcoxon rank-sum tests were used to compare concentrations of innate factors between infants who did versus did not seroconvert to RV5.

To evaluate the association between the combined effect of innate factors and seroconversion, 2 composite scores were developed. The "innate factor" score, of 0 to 3, was generated by adding 3 points for each innate factor concentration (lactoferrin, lactadherin, and TNC) in the highest quartile, 2 points for each innate factor concentration in the second highest quartile, and 1 point for each innate factor concentration in the third highest quartile (no points were assigned for innate factor concentrations in the lowest quartile), and then dividing this sum by 3 to generate a mean. The "innate and acquired immunity" score, of 0 to 6, is a composite score in which innate immunity contributes 50% of the score and acquired immunity (breast milk rotavirus-specific IgA titer) contributes the remaining 50% of the score. This score equals the innate factor score (as stated above) summed with 3 points for a breast milk IgA titer in the highest quartile, 2 points for an IgA titer in the second highest quartile, or 1 point for an IgA titer in the third highest quartile. Two-sided exact Wilcoxon rank-sum tests were used to compare these scores between infants who did versus did not seroconvert to RV5.

RESULTS

Of the 49 mother-infant pairs enrolled, 45 completed study requirements, including 2 infant blood draws and receipt of RV5 by the infant. The 45 infants were 47% male, had a median birthweight of 3200 grams, and had a median age of 2 months of age upon study entry and 3 months of age at the time of the second serum collection. Fifty-three percent lived in the primarily urban region of Perla Maria and 47% lived in Subtiava, which included both urban and periurban regions. Mother-infant pairs lived in households with the following characteristics: 96% had municipal piped water, 71% had an indoor toilet, and 80% had nondirt floors. Infants in the study breastfed a median number of 10 times per day; 29% were exclusively breastfed. Breastfeeding was observed at the immunization visit, on average, within 8 minutes before or after receipt of RV5 (range, 0–43 minutes).

Sixty-nine percent (31 of 45) of infants met the seroconversion definition after the first dose of RV5. Breast milk from mothers had a median lactoferrin concentration of 811.4 μg/mL (interquartile range [IQR], 669.2–1404.2), a median lactadherin concentration of 5.4 μg/mL (IQR, 4.0–7.3), a median TNC concentration of 9.7 μg/mL (IQR, 4.9–16.4), and, as reported previously [13], median rotavirus-specific IgA titers of 160 (IQR, 160–320), and median rotavirus-specific neutralizing antibody titers of 1 (IQR, 1–4).

Lactoferrin concentration in breast milk was moderately correlated with neutralizing antibody titer and rotavirus-specific IgA titer in breast milk ($\rho = 0.66$ and $\rho = 0.54$, respectively; $P < .001$). Lactadherin concentration in breast milk was moderately correlated with breast milk neutralizing antibody titer ($\rho = 0.42$; $P < .001$), but it did not show evidence of correlation with breast milk rotavirus-specific IgA titers ($P = .25$). TNC concentration in breast milk was moderately correlated with breast milk rotavirus-specific IgA titer ($\rho = 0.34$; $P = .02$), but it did not show evidence of correlation with breast milk neutralizing antibody

Table 1. Differences in Concentrations of Immune Factors in Breast Milk of Mothers Whose Infants Seroconverted vs Did Not Seroconvert to the First Dose of Pentavalent Rotavirus Vaccine (Medians [Interquartile Ranges])

Immune Measure*	Seroconverters† (n = 31)	Nonseroconverters (n = 14)	*P* Value‡
Lactoferrin concentration	825.4 [695.6–1413.5]	739.7 [611.7–1143.5]	.71
Lactadherin concentration	5.2 [4.0–8.0]	5.7 [3.8–7.2]	.85
Tenascin-C concentration	9.7 [5.1–17.1]	10.4 [3.7–15.4]	.79
"Innate immunity" score	2.3 [1.6–2.6]	2.1 [1.3–2.6]	.82
"Innate and acquired immunity" score	4.3 [2.6–5.3]	4.3 [3.3–5.6]	.41

*In breast milk.
†Seroconversion defined as a 4-fold or greater increase in rotavirus-specific immunoglobulin A titers in the infant's postdose 1-serum sample compared with the preimmunization sample.
‡Two-sided exact Wilcoxon rank-sum test.

titer ($P = .18$). Concentrations of the innate factors were moderately correlated with each other (lactoferrin and lactadherin, $\rho = 0.62$; lactoferrin and TNC, $\rho = 0.46$; lactadherin and TNC, $\rho = 0.45$ [$P < .001$]).

When examined individually, there were no statistically significant differences in concentrations of breast milk lactoferrin, lactadherin, or TNC concentrations between the groups of infants who did vs did not seroconvert to the first dose of RV5 (Table 1). There were also no statistically significant differences between the innate factor score or innate and acquired immunity score of breast milk between the groups of infants who did versus did not seroconvert to RV5 (Table 1). Furthermore, there was no correlation between either score and fold-change in infant rotavirus-specific IgA titer.

DISCUSSION

Oral vaccines provided to breastfed infants could potentially be inhibited by immune factors in breast milk, a concern raised by in vitro and postlicensure studies [9, 10]. In a prior study, we did not find a clear association between acquired immunity in breast milk and RV5 seroconversion in infants [13]. In this study, we further examined the relationship between innate antiviral factors in breast milk and infant RV5 seroconversion, both individually and as combined scores. Although we did find an association between (1) the concentrations of lactoferrin and lactadherin and (2) breast milk neutralization of rotavirus, we did not find a relationship between concentrations of these innate antiviral factors in breast milk and infant seroconversion to the first dose of RV5. Of note, this was the first study to combine concentrations of innate factors into scores to examine their combined effect. In contrast with the lack of association of breast milk immunity with seroconversion, as we reported previously, high maternal serum humoral immunity appears to most strongly predict failure to seroconvert to RV5 [13], suggesting potential interference by placentally transferred maternal IgG with infant response to RV5.

However, we did find that the concentrations of each of these innate factors in breast milk were moderately correlated to each other, suggesting that the concentration of these proteins is predicted by total milk protein content. In addition, as lactoferrin and lactadherin concentrations were moderately correlated with neutralizing antibody titers in breast milk, this suggests that they may serve a role in protecting the infant against natural rotavirus infection.

Although this study was limited by a small sample size, our findings do not support the withholding of breastfeeding at the time of RV administration as a potential intervention to improve infant RV response. Two prior studies have examined the effect of breastfeeding withholding at the time of RV administration, and neither found a benefit in terms of improved immunogenicity [11, 12].

CONCLUSIONS

In conclusion, this preliminary study found that innate antiviral factors in breast milk are not associated with RV5 seroconversion in infants. Future research should focus on maternal IgG antibody interference and other host factors in LMIC infants to better understand and develop strategies to improve the effectiveness of RVs in these settings.

Notes

Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the official positions of the Centers for Disease Control and Prevention.

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