Mastitis and Immunological Factors in Breast Milk of Lactating Women in Malawi

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Although an elevated sodium concentration in human milk is suggested to be an indicator of mastitis, it is unclear whether elevated sodium concentrations are associated with immunological and inflammatory mediators in human milk. We conducted a cross-sectional study to evaluate the relationships between elevated breast milk sodium concentrations and levels of lactoferrin, lysozyme, secretory leukocyte protease inhibitor (SLPI), interleukin-8 (IL-8), and RANTES (regulated on activation normal T cell expressed and secreted) in human milk at 6 weeks postpartum in 96 lactating women in Blantyre, Malawi. Mastitis, as indicated by an elevated breast milk sodium concentration, was present in 15.6% of the women. Women with and without mastitis had respective median levels of other factors as follows: lactoferrin, 1,230 versus 565 mg/liter (P < 0.0007); lysozyme, 266 versus 274 mg/liter (P = 0.55); SLPI, 76 versus 15 mg/liter, (P < 0.0002); IL-8, 339 versus 25 ng/liter (P < 0.0001); and RANTES, 82 versus 3 ng/liter (P < 0.0001). Elevated sodium concentrations in breast milk are associated with an increase in levels of some immunological and inflammatory factors in breast milk.

Recent large epidemiological studies demonstrate that clinically apparent mastitis affects about 20 to 30% of breastfeeding women (10, 15, 17, 27). Mastitis, if untreated, can lead to lactation failure, recurrent mastitis, or breast abscesses (18). The early diagnosis and treatment of mastitis may help prevent more serious supplicative infection, recurrent mastitis, and other complications. In normal human milk, sodium concentrations are about 5 to 6 mmol/liter, and the sodium concentration in breast milk is tightly regulated (1, 2, 16, 33). Sodium concentrations in mature human milk (2 weeks postpartum) are thought to be low because milk is separated from other fluids by tight junctions between mammary alveolar cells. However, during mastitis, inflammatory cells enter the milk, and inflammation is accompanied by an opening of tight junctions, which allows intercellular fluid and plasma to enter the milk through paracellular pathways between alveolar cells (23, 25). Elevated breast milk sodium concentrations are considered to be sensitive indicators of mastitis (8, 11, 12, 23, 25), and are used to detect subclinical mastitis in animals (35). It is unclear whether elevated sodium concentrations in human milk are associated with increased concentrations of immunological and inflammatory mediators in breast milk.

Human milk contains important immunological factors which are considered to be protective against infection and may modulate inflammation in the mammary alveoli. Lactoferrin, a 703-amino-acid glycoprotein, is found in high concentrations in human milk and has both bacteriostatic and bactericidal actions (5). Lysozyme, a 12-kDa single-chain protein, lyzes susceptible bacteria by cleaving peptidoglycans of bacterial cell walls (7). Secretory leukocyte protease inhibitor (SLPI), an 11.7-kDa serine protease inhibitor, protects tissues from degradation by proteases which are released by neutrophils, such as elastase and cathepsin G (31). Two chemokines, interleukin-8 (IL-8) and RANTES (regulated on activation normal T cell expressed and secreted), are found in relatively high concentrations in human milk (20). IL-8 is produced in response to inflammatory stimuli and plays a role in the recruitment and activation of neutrophils and lymphocytes (3). RANTES, a chemokine produced by CD8+ lymphocytes, natural killer cells, and mammary epithelial cells, is involved in the chemotaxis of macrophages (34). During clinically apparent mastitis, lactoferrin, lysozyme, and IL-8 levels are known to increase in human and bovine milk (4, 13, 25, 26).

We hypothesized that lactoferrin, lysozyme, SLPI, IL-8, and RANTES concentrations in human milk are higher among women with mastitis, as indicated by elevated human milk sodium concentrations, than among women without mastitis. To examine this hypothesis, we measured concentrations of breast milk sodium and the aforementioned immunological factors in milk obtained at 6 weeks postpartum from women in Blantyre, Malawi.

MATERIALS AND METHODS

The study population consisted of 96 lactating women who were seen at the Queen Elizabeth Central Hospital in Blantyre, Malawi, from March 1996 through May 1997. Women were seen during the second trimester of pregnancy and enrolled in the study after having given written, informed consent. The study protocol was approved by the ethical review committees at Johns Hopkins University, the University of Malawi, and the Ministry of Health, Government of Malawi. All women in the study received counseling and testing for sexually transmitted diseases (STDs) and human immunodeficiency virus (HIV) infection, physical examination, and treatment of STDs, malaria, and iron deficiency anemia in the clinic. Height and weight were recorded. Women were tested for the presence of HIV antibody in serum by using enzyme-linked immunosorbent assay (ELISA) (Wellcozyme [Wellcome Diagnostics, Dartford, Kent, United Kingdom]) and enzyme immunoassay [Genetic Systems, Seattle, Wash.]). HIV-positive women were not included in this study. Following delivery, women in the study continued to receive counseling and testing for STDs, physical examination, and treatment of STDs, malaria, and iron deficiency anemia at each visit. Antibiotic treatment was given to women with clinically apparent mastitis, but...
TABLE 1. Characteristics of women with and without mastitis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (values for the 25th and 75th percentiles)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>Age (yr) 21 (16, 26)</td>
<td>0.11</td>
</tr>
<tr>
<td>Body mass index (wt/ht&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>Body mass index 23.0 (21.5, 25.3)</td>
<td>0.42</td>
</tr>
<tr>
<td>Birth wt (g)</td>
<td>Birth wt 2,950 (2,550, 3,115)</td>
<td>0.16</td>
</tr>
<tr>
<td>Concen in breast milk of:</td>
<td>Concen in breast milk 1,230 (983, 1,731)</td>
<td></td>
</tr>
<tr>
<td>Lactoferrin (mg/liter)</td>
<td>Lactoferrin 565 (443, 778)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Lysozyme (mg/liter)</td>
<td>Lysozyme 274 (137, 389)</td>
<td>0.55</td>
</tr>
<tr>
<td>SLPI (µg/liter)</td>
<td>SLPI 17 (10, 28)</td>
<td>0.0002</td>
</tr>
<tr>
<td>IL-8 (ng/liter)</td>
<td>IL-8 25 (0, 63)</td>
<td>0.0001</td>
</tr>
<tr>
<td>RANTES (ng/liter)</td>
<td>RANTES 3 (0, 14)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Laboratory diagnosis of mastitis based upon a breast milk sodium concentration of >12 mmol/liter.

<sup>b</sup> Determined by the Wilcoxon rank-sum test.

clinical records (for the involved breast) could not be linked with breast milk samples (from either breast) in this a posteriori study. At 6 weeks postpartum, a sample of milk was obtained randomly from either breast by manual expression, and breast milk was immediately aliquoted and stored in a sample archive at −70°C until analysis for sodium, potassium, lactoferrin, lysozyme, SLPI, IL-8, and RANTES concentrations was conducted. Breast milk samples were centrifuged at 1,300 × g for 7 min, and the lipid and aqueous portions were separated and removed. The aqueous portion was analyzed for sodium and potassium concentrations by using ion-selective electrodes on a Boehringer Mannheim/Hitachi 747 Analyzer (Roche/Boehringer Mannheim, Indianapolis, Ind.) in the Department of Pathology, the Johns Hopkins Hospital. Breast milk sodium levels were defined as elevated at concentrations of >12 mmol/liter, because this concentration of sodium is more than 3 standard deviations above the mean for normal human milk at 1 month of lactation, determined by using ion-selective electrodes on fat-free samples (9, 22). It should be noted that the older literature indicates that atomic absorption photometry has yielded higher ranges for human milk sodium concentrations than those obtained with ion-selective electrodes. In this paper, “women with mastitis” refers to women who had elevated breast milk sodium concentrations consistent with mastitis unless otherwise indicated.

Lactoferrin in human milk was measured by using a sandwich ELISA with plates coated with rabbit anti-human lactoferrin (Organon Teknika, Durham, N.C.), dilutions of human breast milk, peroxidase-conjugated rabbit immunoglobulin G to human lactoferrin (Organon Teknika), and substrate development with 3-phenylenediamine dihydrochloride. Absorbances were read at 490 nm, with wavelength correction at 630 nm. Purified human lactoferrin (Organon Teknika) was used as a standard to estimate the amount of breast milk lactoferrin. Lysozyme in human milk was measured by radial immunodiffusion assay (Nanorid, human lysozyme; The Binding Site, Birmingham, United Kingdom). SLPI, IL-8, and RANTES levels in human milk were measured by using ELISA (Quantikine, Human SLPI Immunoassay, Human IL-8 Immunoassay, and Human RANTES Immunoassay; R & D Systems, Minneapolis, Minn.).

The Wilcoxon rank-sum test was used for nonparametric comparison of continuous variables between groups. Chi-square tests or Fisher’s exact tests were used to compare categorical variables between groups. Spearman correlation was used to examine the relationship between sodium concentrations, sodium/potassium ratios, and levels of breast milk immunological factors.

RESULTS

Elevated human milk sodium concentrations consistent with mastitis (>12 mmol/liter) were present in 15 of 96 (15.6%) lactating women at 6 weeks postpartum. Among the 96 lactating women in the study, median concentrations (with the values for the 25th and 75th percentiles in parentheses) of breast milk immunological and inflammatory mediators were as follows: lactoferrin, 616 (464, 963) mg/liter; lysozyme, 267 (151, 397) µg/liter; SLPI, 19 (11, 34) µg/liter; IL-8, 28 (1, 78) pg/ml; and RANTES, 5 (0, 23) pg/ml. Women with mastitis had significantly higher levels of lactoferrin, SLPI, IL-8, and RANTES in their breast milk than women who did not have mastitis (Table 1). There were no significant differences in lysozyme levels, age, and body mass index between women with and without mastitis.

Spearman correlation was used to examine the relationship between sodium levels, the sodium/chloride ratio, and levels of lactoferrin, lysozyme, SLPI, IL-8, and RANTES in breast milk (Table 2). The Spearman rank correlation coefficient was >0.40 between sodium and each immunological factor in breast milk except lysozyme. The correlation with immunological factors in breast milk was lower for the sodium/potassium ratio than for sodium.

DISCUSSION

This study suggests that at 6 weeks postpartum, about 16% of lactating women had elevated sodium concentrations in breast milk which were consistent with mastitis. These findings are consistent with recent, large epidemiological studies which suggest that clinically apparent mastitis occurs in about 20 to 33% of lactating women at some time during lactation, mostly within the first 2 months after delivery. A prospective cohort study of 1,075 women in Australia found a crude incidence rate of mastitis of 20% during the first 6 months after delivery (17). A study of American women suggested that about one-third of women who breastfeed develop mastitis (27). In Finland, 24% of 664 breastfeeding women developed mastitis (15). Another

TABLE 2. Spearman rank correlation coefficients for factors in human milk

<table>
<thead>
<tr>
<th>Factor (n)</th>
<th>Na/K ratio</th>
<th>Lactoferrin</th>
<th>Lysozyme</th>
<th>SLPI</th>
<th>IL-8</th>
<th>RANTES (n = 95)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na (96)</td>
<td>0.908 (0.0001)</td>
<td>0.459 (0.0001)</td>
<td>0.133 (0.19)</td>
<td>0.429 (0.0001)</td>
<td>0.520 (0.0001)</td>
<td>0.430 (0.0001)</td>
</tr>
<tr>
<td>Na/K ratio (96)</td>
<td>0.268 (0.02)</td>
<td>0.060 (0.56)</td>
<td>0.221 (0.04)</td>
<td>0.423 (0.0001)</td>
<td>0.319 (0.002)</td>
<td>0.489 (0.0001)</td>
</tr>
<tr>
<td>Lactoferrin (75)</td>
<td>0.179 (0.12)</td>
<td>0.713 (0.0001)</td>
<td>0.518 (0.0001)</td>
<td>0.311 (0.0005)</td>
<td>0.193 (0.06)</td>
<td>0.486 (0.0001)</td>
</tr>
<tr>
<td>Lysozyme (95)</td>
<td>0.254 (0.02)</td>
<td>0.863 (0.0001)</td>
<td>0.533 (0.0001)</td>
<td>0.653 (0.0001)</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>SLPI (92)</td>
<td>0.486 (0.0001)</td>
<td>0.653 (0.0001)</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8 (95)</td>
<td>0.486 (0.0001)</td>
<td>0.653 (0.0001)</td>
<td>0.0001</td>
<td></td>
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</tbody>
</table>
prospective cohort study of 306 breastfeeding mothers showed that 27% developed mastitis within 3 months of delivery (10). Earlier studies may have underestimated the incidence of mastitis, as these investigations relied upon the presentation of women with mastitis at hospitals and many women seek attention from midwives and general practitioners outside the hospital setting (17, 27). The incidence of mastitis is highest within the first 2 months after delivery (10, 17, 24).

Elevated sodium concentrations in human milk were associated with elevated concentrations of lactoferrin, SLPI, IL-8, and RANTES. These data support the idea that elevated sodium concentrations in breast milk are associated with inflammation in mammary alveoli. Both lactoferrin and lysozyme levels are known to increase in milk during clinical mastitis (25). Among cells which produce lactoferrin are neutrophils and mammary epithelial cells (21). Median lactoferrin levels in milk were about twice as high in women with elevated breast milk sodium concentrations as in women with normal breast milk sodium concentrations; however, there were no significant differences in median lysozyme levels between women with and without elevated breast milk sodium levels.

Lysozyme levels were significantly correlated with SLPI and IL-8 levels but had poor correlation with sodium levels. It may be possible that this study did not detect elevated lysozyme levels in breast milk because of limited sample size or because the changes in lysozyme levels with mastitis occur with a time course different from that of other mediators in human milk. The small sample size of the present study gave a 95% confidence interval that the median lysozyme concentration for women with mastitis was 0.79 to 2.07 times the median lysozyme concentration for women without mastitis.

The levels of lactoferrin in human milk in the present study were lower than those reported from the Gambia (25) and Bangladesh (11) but are closer to the ranges reported from Zaire (14). The differences in these measured levels of lactoferrin may be due to differences in the methods and standards which were used (radioimmunoassay versus ELISA) in the studies. It is unclear whether nutritional status affects the levels of lactoferrin and lysozyme in breast milk. The levels of lysozyme in human milk reported in the present study were higher than those reported from the Gambia (25) and Bangladesh (11) but were closer to the range reported from a study in Zaire (14). Different studies have used radial immunodiffusion, radioimmunoassay, and ELISA for measurement of lysozyme in human milk, and differences in these methods may have accounted for the contrasting levels of lysozyme found in the studies.

SLPI is the dominant protease inhibitor produced by cells on a wide variety of mucosal surfaces, such as the parotid gland, lung, and cervix. During inflammation SLPI appears to defend host tissues against degradation by tissue-destructive proteases released by polymorphonuclear leukocytes. Median SLPI levels were about five times higher in women with elevated breast milk sodium concentrations than in women with normal breast milk sodium concentrations, and the correlation between SLPI and lactoferrin was high. These data suggest that SLPI is produced in the mammary alveoli in response to inflammation, probably by the mammary epithelial cells.

Human milk contains high concentrations of IL-8, a chemotactic cytokine involved in the migration of neutrophils and lymphocytes (4, 20, 30). Immunohistochemistry demonstrates reactivity for IL-8 and RANTES in the acinar epithelial cells of normal mammary tissue (20). Median IL-8 levels were about 13 times higher among women who had elevated breast milk sodium concentrations than in women with normal sodium concentrations, and IL-8 levels were significantly correlated with levels of sodium and immunological factors measured in breast milk. A bovine mammary epithelial cell line produces IL-8 in response to lipopolysaccharide stimulation in vitro (6), suggesting that mammary epithelial cells secrete IL-8 in response to infectious stimuli. Mastitic breast milk contains levels of IL-8 which elicit the chemotaxis of neutrophils (4). RANTES, a chemokine involved in the chemotaxis of monocytes, eosinophils, and memory CD4+ lymphocytes, has been detected in human milk (20). In this study, women with elevated breast milk sodium concentrations had median RANTES levels which were about 27 times higher than those in women with normal breast milk sodium concentrations. There were high and significant correlations between RANTES levels and sodium, lactoferrin, SLPI, and IL-8 levels in breast milk.

A limitation of this study is that microbiological cultures of breast milk and leukocyte counts were not done; however, correlation between elevated breast milk sodium levels and clinically apparent mastitis has been made elsewhere (8, 23). Other causes of increased breast milk sodium levels include early weaning and poor feeding by a malnourished and dehydrated infant. These causes seem unlikely in the present study, as all women were exclusively breastfeeding at 6 weeks postpartum, and none of the infants were noted to be malnourished or dehydrated at the time of examination. The present study provides further corroborating evidence that elevated breast milk sodium concentrations are associated with elevated levels of inflammatory and immunological mediators such as lactoferrin, SLPI, IL-8, and RANTES. Breast milk sodium concentrations alone had better correlation with factors in breast milk than the sodium/potassium ratio, suggesting that sodium alone might be a more accurate indicator for mastitis.

Sodium concentrations can be measured quickly by using simple and relatively inexpensive desktop electrolyte analyzers, and further studies are needed to validate use of such tools for screening of mastitis in a clinical setting.

A recent study shows that mastitis, as indicated by elevated breast milk sodium concentrations, is associated with a higher HIV load in milk and higher mother-to-child transmission of HIV (28, 29), and these observations emphasize the potential importance of screening and treatment of mastitis. At 6 weeks postpartum, the proportion of HIV-infected women with elevated breast milk sodium concentrations was about 16%, a proportion similar to that found among HIV-negative women in the present study. There are very few studies regarding the microbiology of human mastitis in the literature (19, 24, 32), and much work is needed to characterize the microbiological pathogens involved in mastitis in developing countries.

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