

Evidence for Long-Term Memory of the Mucosal Immune System: Milk Secretory Immunoglobulin A against *Shigella* Lipopolysaccharides

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Although the common mucosal immune system has generally been considered to have only short-term memory, recent data suggest that long-term memory exists for *Shigella* virulence plasmid antigens. Because such antigens might cross-react with environmental antigens, we investigated milk for the persistence of antibodies to the specific *Shigella* lipopolysaccharide (LPS) antigens. Enzyme-linked immunosorbent assays to detect secretory immunoglobulin A (sIgA) against *Shigella flexneri* and *Shigella sonnei* LPS in milk samples were developed; 15 random milk samples tested on different days correlated from one day to the next ($P = 0.0001$). Of 18 Mexican mothers, 18 (100%) had one or more milk samples positive for anti-*S. flexneri* LPS, 14 (78%) had one or more milk samples positive for anti-*S. sonnei* LPS, and 14 (78%) had one or more milk samples positive for both. Of 27 Houston mothers, 16 (59%) had one or more milk samples positive for anti-*S. flexneri* LPS, 7 (26%) had one or more milk samples positive for anti-*S. sonnei* LPS, and 5 (19%) had one or more milk samples positive for both. Mexican mothers were significantly more likely than Houston mothers to have at least one sample with a positive titer for anti-*S. flexneri* LPS ($P < 0.002$) and at least one sample with a positive titer for anti-*S. sonnei* LPS ($P < 0.002$). Although the Houston women had a lower rate of titer positivity for both *Shigella* species, the rate was too high to be consistent with short-lived mucosal immunity. It is unlikely that 18 of the 27 Houston women had shigellosis during or just prior to lactation. The data suggest that there exists a long-term hormonally driven memory in the secretory immune system for *Shigella* spp.

Shigella spp. and other enteric pathogens are major causes of morbidity and mortality in infants and young children, especially in developing countries. Breastfeeding appears to play an important role in protecting infants from symptomatic gastrointestinal infections. Several studies have shown that the number and severity of *Shigella* infections (7, 19, 25, 26, 41) are decreased in children who are breastfed compared with those who are formula fed. Information gained by studying the mechanisms by which human milk protects against infection with *Shigella* spp. and other enteric pathogens may be useful in designing and evaluating future *Shigella* vaccines.

To date, the exact mechanisms by which breastfeeding is protective remain unclear. In addition to providing the infant with an uncontaminated source of nutrition, breast milk contains phagocytic cells (neutrophils and macrophages), B cells, T cells, specific antibodies, and nonspecific cell receptor analogs that react with bacterial virulence factors (4, 12, 32, 33, 36, 37, 40, 43).

In breast milk, the presence of secretory immunoglobulin A (sIgA) antibodies directed against specific enteric pathogens has been shown and is thought to be a result of the migration of gut-derived lymphocytes to the mammary tissues (28–31, 40). It is not known exactly how the mammary tissue preferentially attracts IgA-producing B cells (31). The migration of IgA-producing plasma cells and the synthesis of sIgA in the mammary gland are governed by the hormones of pregnancy and lactation (34).

The patterns of anti-*Shigella* lipopolysaccharide (LPS)

antibody excretion in human milk over the course of lactation have not been extensively studied. Previous studies have documented that human milk sometimes contains sIgA to *Shigella* LPS (1, 11, 21). Little is known about the timing of primed lymphocyte migration to the mammary tissues or the duration of memory for these cells. We, therefore, evaluated serial milk samples of women from two populations of differing *Shigella* incidence for sIgA antibodies directed against the LPS of *Shigella flexneri* and the LPS of *Shigella sonnei* to gain some insight into the dynamics of the secretory immune system as it relates to *Shigella* spp. and breast milk.

Patient population and collection of milk samples. Milk samples were collected from 18 Mexican women living in an urban area on the outskirts of Mexico City and from 27 women living in Houston, Texas. The Houston subjects included 21 white (non-Hispanic) women, 3 black women, and 1 Asian-Indian woman. Data on the race of the remaining two women from Houston were not available. It was not known whether the individual women had experienced diarrhea due to *Shigella* infection prior to lactation. Milk samples were collected on various days of lactation (range, day 3 to 247 of lactation) by means of an Egnell breast pump with polypropylene containers; samples were stored at -20°C . Each woman submitted an average of four milk samples (range, two to nine samples). Blood and stool samples were not available for analysis. Milk samples were thawed and centrifuged in an Eppendorf rotor ($12,000 \times g$ for 15 min), and the upper layer of fat and the residual pellet were removed. The remaining clear-liquid fraction was respun ($12,000 \times g$ for 15 min), and, again, the fat and residual pellet

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were removed. Spun milk samples were diluted in 3% nonfat dry milk at dilutions of 1:20 to 1:40,960.

Development of anti-*S. flexneri* and anti-*S. sonnei* LPS enzyme-linked immunosorbent assays (ELISAs). LPS was purified from *S. flexneri* serotype 5, strain M90T (38), and *S. sonnei* UT-15 (45) by using the hot phenol-water extraction method of Westphal and Jann (44). The resulting LPS preparations were stored at 4°C and later diluted in either phosphate-buffered saline (PBS) or Tris buffer. The presence of purified LPS was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for both the *S. flexneri* and *S. sonnei* preparations. Staining of the gels with periodic acid and silver stains showed the characteristic stepladder patterns expected for *S. flexneri* LPS and *S. sonnei* LPS. When both preparations were analyzed by SDS-PAGE and stained with Coomassie blue stain, there were no protein bands to suggest the presence of protein contaminants in the LPS preparations.

An ELISA for sIgA against LPS was carried out as follows. The wells of Immulon II U-bottom plates (Dynatech Labs, Inc., Chantilly, Va.) were coated with 100 µl of 0.05 M sodium carbonate buffer (pH 9.6) or with *S. sonnei* LPS or *S. flexneri* LPS (10 µg/ml) in the same buffer. The plates were incubated overnight at 4°C, washed twice with PBS containing 0.05% Tween 80 (PBST), and blocked with 3% nonfat dry milk at 37°C for 1 h. Milk samples at various dilutions were then incubated at 37°C for 1 h. The wells were then washed four times with PBST, and goat anti-human sIgA conjugated to horseradish peroxidase (Cappel, Philadelphia, Pa.), at a dilution of 1:40,000 in nonfat dry milk, was added to each well. After a 1-h incubation period at 37°C, the plates were washed four times with PBST, and a solution of peroxidase and *O*-phenylene-diamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) in citrate buffer was used to develop the reaction (100 µl per well). The reaction was stopped with 2.5 N H₂SO₄, and the plates were read on an automatic ELISA reader (Dynatech Labs) at 470 nm.

Antibody titers were determined as follows. For each milk dilution, the reading for the sample in a well without LPS (i.e., a well coated only with carbonate buffer) was subtracted from the reading for the same sample in a well coated with LPS. All samples were done in duplicate at all dilutions. A known positive milk sample and a known negative milk sample were run as positive and negative controls on each plate, and nonfat dry milk alone was run as an additional negative control on each plate. The positive and negative control milk samples had been previously confirmed by Western blot (immunoblot). The milk sample which was the positive control for the anti-*S. flexneri* LPS ELISA was the negative control for the anti-*S. sonnei* LPS ELISA. If the difference in readings was ≥ 0.100 , the sample was considered positive at that dilution; the cutoff used to determine a positive sample was calculated as the reading which was 3 standard deviations above the mean of three negative control samples. This calculated cutoff was consistently found to be very close to 0.100 U; thus, for convenience, 0.100 U was the cutoff used on all plates.

Reproducibility of ELISAs. The ELISAs for sIgA anti-*S. flexneri* LPS and sIgA anti-*S. sonnei* LPS were shown to have good reproducibility. When 15 random milk samples were tested on two different days, all resulting titers were identical except for four samples tested on the *S. flexneri* ELISA and two samples tested on the *S. sonnei* ELISA, which differed by only one tube dilution. Regression analysis showed a good correlation between the two ELISA tests (for

TABLE 1. Comparison of anti-*Shigella* LPS positivity in Houston and Mexican women

Women from:	No. of women (%)			
	Anti- <i>S. flexneri</i> LPS ^a		Anti- <i>S. sonnei</i> LPS ^b	
	All milk samples negative	At least one milk sample positive	All milk samples negative	At least one milk sample positive
Houston	11 (41)	16 (59)	20 (74)	7 (26)
Mexico	0 (0)	18 (100)	4 (22)	14 (78)

^a $P < 0.002$.

^b $P < 0.002$.

anti-*S. flexneri* LPS, $r = 0.88$, $P < 0.01$; for anti-*S. sonnei* LPS, $r = 0.98$, $P < 0.02$).

Comparison of anti-*Shigella* LPS positivity of Mexican and Houston women. Of the 18 women from Mexico, 18 (100%) had at least one milk sample positive for sIgA against *S. flexneri* LPS, 14 (78%) had at least one milk sample positive for sIgA against *S. sonnei* LPS, and 14 (78%) had one or more milk samples positive for both. Of the 27 women from Houston, 16 (59%) had at least one milk sample positive for sIgA against *S. flexneri* LPS, 7 (26%) had at least one milk sample positive for sIgA against *S. sonnei* LPS, and 5 (19%) had one or more milk samples positive for both. When these data were compared by using contingency table analysis, it was found that Mexican women were significantly more likely than Houston women to have at least one milk sample with a positive titer for anti-*S. flexneri* LPS ($P < 0.002$), and the same was true for anti-*S. sonnei* LPS antibodies ($P < 0.002$) (Table 1). Four of 18 (22%) Mexican women and 13 of 27 (48%) Houston women had milk samples which were positive for sIgA antibodies against one but not the other *Shigella* LPS.

Evaluation of sIgA titers over lactation time. Titers of colostrum ranged from 0 to 1:1,280 for anti-*S. flexneri* LPS and 0 to 1:5,120 for anti-*S. sonnei* LPS; titers of mature milk ranged from 0 to 1:640 for anti-*S. flexneri* LPS and from 0 to 1:320 for anti-*S. sonnei* LPS for all women who did not report diarrhea during lactation. The geometric mean titers of all available milk samples from women who did not report diarrhea during lactation are plotted against days of lactation in Fig. 1 and 2. There were women from both Mexico and Houston who showed persistently positive titers for anti-*S. flexneri* LPS or anti-*S. sonnei* LPS as late as the eighth month of lactation. In the Houston population where reinfection with *Shigella* spp. during lactation is generally a rare event, 10 of 18 (56%) women who had positive titers for either anti-*S. flexneri* LPS, anti-*S. sonnei* LPS, or both, continued to have persistently positive titers throughout the course of lactation (as late as day 247), demonstrating presumably hormonally driven antibody secretion. In general, most Houston women (23 of 27 or 85%) did not develop significant increases in anti-*S. sonnei* LPS or anti-*S. flexneri* LPS titers throughout the course of lactation; however, a dramatic exception was seen in a Houston woman who had an illness thought to be shigellosis. Her titers for sIgA against *S. sonnei* LPS rose from 1:20 early in lactation to 1:5,120 on day 94 and remained persistently elevated at $\geq 1:640$ for the remainder of lactation; she reported that she had diarrhea and fever for 3 days at the end of the second month of lactation, but no stool culture had been performed

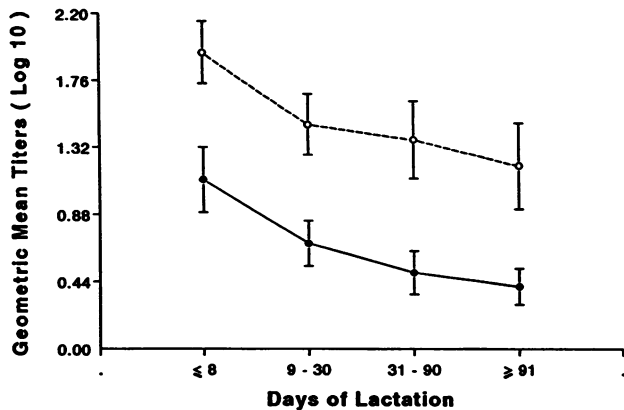


FIG. 1. Geometric mean titers of sIgA anti-*S. flexneri* LPS of all available milk samples from women who did not report diarrhea during lactation. Symbols: ○, 54 milk samples from 18 Mexican women; ●, 109 milk samples from 25 Houston women. The Mexican milk samples included 17 in the ≤8-day group, 19 in the 9- to 30-day group, 10 in the 31- to 90-day group, and 8 in the ≥91-day group; the Houston samples included 15, 31, 22, and 41 in each of these groups, respectively.

during this illness to confirm the diagnosis of *S. sonnei* infection.

Discussion. The immune responses to *Shigella* infection are complex. Many animal and human studies have been done evaluating the anti-*Shigella* immunoglobulin levels of various body fluids after both natural infection and vaccination. Studies of anti-*Shigella* antibodies in serum have shown that the immunoglobulins produced after acute, natural infection are of the IgA, IgM, and IgG classes. Most studies have evaluated the antibodies directed against the specific LPS of *Shigella* spp. (8, 9, 14-16, 35). In a study by Cohen et al., significant serum antibody responses measured by ELISA were detected in 73 to 82% of symptomatic adults and in 48 to 60% of asymptomatic adults during several *S. flexneri* and *S. sonnei* outbreaks. The anti-*Shigella* LPS IgA levels in particular were highest 2 weeks after infection and declined to initial levels within 2.5 months (8). Persson et al. showed that serum IgA against *S. flexneri* LPS peaked

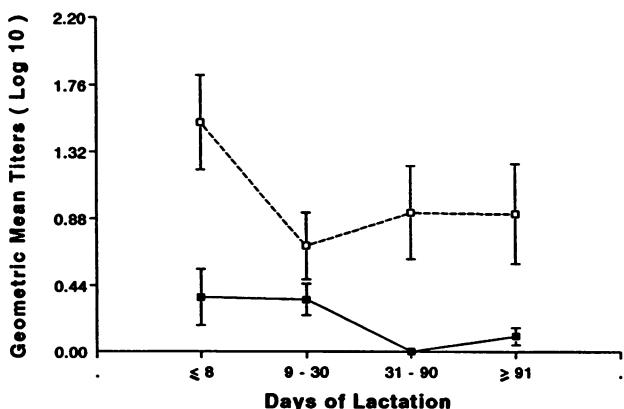


FIG. 2. Geometric mean titers of sIgA anti-*S. sonnei* LPS of all available milk samples from women who did not report diarrhea during lactation. Symbols: □, 54 milk samples from 18 Mexican women; ■, 109 milk samples from 25 Houston women. The samples described in the legend to Fig. 1 are shown.

within 4 weeks after infection, but the duration of anti-*Shigella* LPS antibody positivity was not extensively evaluated in his group of 17 adult Swedish patients (35). In one study of 97 adult and pediatric patients with *S. flexneri* infection in Hanoi, Vietnam, children 1 to 2 years of age were found to have elevated serum IgA titers against *S. flexneri* LPS up to 60 days after falling ill (14). In a study of 14 Swedish adults with *S. flexneri* dysentery, 79% (11 of 14) responded with positive serum IgA titers against *S. flexneri* LPS within 4 weeks of the onset of infection and as many as 56% showed persistently positive IgA titers at 1 year post-infection (16). Serum studies of IgA directed against the virulence plasmid-associated antigens have also been done. In a study of 41 Thai adults and children, 8 of 19 children and 1 of 22 adults were shown to develop IgA antibodies against virulence plasmid-associated antigen 3 to 4 weeks after acute shigellosis (13). Thus, it is apparent that the serum IgA response to *Shigella* infection is immediate, peaks at 2 to 4 weeks after infection, and drops to low levels within 2.5 months.

Studies of anti-*Shigella* antibodies in stool have been carried out by using Western blotting and dot blotting techniques. In one study, increases in fecal IgA against *S. flexneri* water extracts and *S. sonnei* LPS were seen after 5 days of illness (45). Long-term studies evaluating anti-*Shigella* sIgA in human stool samples have not been reported. Animal data, however, indicate that the local sIgA response in the intestinal tract is not persistent. In rabbit ileal loop studies, sIgA levels initially increased but then decreased or dropped to baseline within 24 to 32 days after challenge with various *Shigella* species (22, 23).

Evaluations of human colostrum and breast milk for IgA antibodies against *Shigella* spp. have also been done. In 1982, Cruz et al. showed that women from differing populations (Sweden and Guatemala) had comparable quantities of milk antibodies to *Escherichia coli* (pool of eight somatic *E. coli* antigens) (10). However, in 1985, the same investigators studied anti-*Shigella* LPS antibodies in the milk of women from the same two geographic areas and found that the concentrations of IgA differed among Swedish, rural and urban poor Guatemalan, and privileged Guatemalan women (11). We have likewise found population-based differences in sIgA antibody titers in breast milk samples.

In this study, we developed two ELISAs which were shown to be reliable and reproducible in measuring anti-*S. flexneri* LPS and anti-*S. sonnei* LPS sIgA titers. There was no cross-reaction between antibodies against *S. flexneri* LPS and *S. sonnei* LPS. This was expected, given the structural dissimilarities for both the O-repeat units and core oligosaccharides produced by these organisms (2, 3, 20, 24, 39). The fact that several women had antibodies to one but not the other LPS and the fact that the sample which was the Western-blot-proven positive control for the *S. flexneri* LPS ELISA was also the negative control for the *S. sonnei* LPS ELISA suggest that the antibodies measured were specific and were made to the specific O side chains of the LPS molecule rather than a shared lipid A component.

The data for individual women are consistent with the concept that when an acute *Shigella* infection occurs during lactation, the sIgA titers of breast milk may rise and persist. In cases where such acute increases in specific antibody are seen, the response may represent either initial exposure to the antigen or an amnesic response triggered by reexposure to a previously recognized antigen. The most dramatic example of this is the history of diarrhea and fever in one of the Houston women during lactation, with a subsequently

documented (>64-fold) increase in breast milk antibody titer to *S. sonnei*. The data demonstrate that Mexican women were significantly more likely to have at least one breast milk sample positive for sIgA antibodies against both *S. flexneri* LPS and *S. sonnei* LPS. This is consistent with the fact that the incidence of shigellosis in the general population is higher in Mexico than it is in Houston. Although the Houston women had a lower rate of titer positivity for both *Shigella* species in this study, the rate was not as low as would be expected if, indeed, mucosal immunity is a short-lived phenomenon, as expected from the fecal sIgA data. It is unlikely that 18 of the 27 Houston women had shigellosis during the months prior to lactation, but it is likely that they had shigellosis at some point during the 20 to 30 years of life prior to lactation. The high frequency of persistence of antibody secretion in the Houston women (10 of 18; 56%) further supports this concept. In addition, the immune responses to these *Shigella* LPS antigens are unlikely to represent responses to normal flora. Antibody to the LPS of *S. flexneri* serotype 5 cross-reacts with only a single *E. coli* O antigen (O-129) (17), and antibody to *S. sonnei* LPS is known to cross-react only with *Pleisimonas shigelloides* type 27C (17, 18). There is no source for repeated antigenic stimulation in the community to boost antibody titers as there is for polio or for the cholera-like toxins of *Campylobacter* spp., *Salmonella* spp., or *E. coli*. Therefore, most of the Houston women with positive titers were presumably manifesting a hormonally driven, rather than an antigen-triggered, antibody secretion. Hormonal control of the common mucosal immune system has previously been demonstrated in both animals and humans. In the ovariectomized rat model, uterine IgA has been shown to be under the control of estradiol (46). Likewise, in the mouse, progesterone, estrogen, and prolactin determine the ability of the mammary gland to trap IgA immunoblasts (42). In humans, it is known that immunoglobulin localization within the mammary gland varies with the menstrual cycle (27).

sIgA antibodies to the polypeptide virulence plasmid-encoded antigens of *Shigella* in human milk also persist throughout lactation (5, 6). Because multiple epitopes exist in the virulence plasmid antigens, cross-reactions with environmental antigens are impossible to exclude. Because reactions to *Shigella* LPSs are unlikely to be induced by cross-reacting antigens, the demonstration of sIgA throughout lactation suggests that there exists a long-term memory in the secretory immune system for *Shigella* spp.

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