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Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants

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To test the hypothesis that human milk fucosyloligosaccharides are part of an innate immune system, we addressed whether their expression (1) depends on maternal genotype and (2) protects breastfed infants from pathogens. Thus the relationship between maternal Lewis blood group type and milk oligosaccharide expression and between variable oligosaccharide expression and risk of diarrhea in their infants was studied in a cohort of 93 Mexican breastfeeding mother–infant pairs. Milk of the 67 Le^{a-b+} mothers contained more LNF-II (Le^a) and 3-FL (Le^x) (oligosaccharides whose fucose is exclusively α1,3- or α1,4-linked) than milk from the 24 Le^{a-b-} mothers; milk from Le^{a-b-} mothers contained more LNF-I (H-1) and 2′-FL (H-2), whose fucose is exclusively α1,2-linked. The pattern of oligosaccharides varied among milk samples; in each milk sample, the pattern was summarized as a ratio of 2-linked to non-2-linked fucosyloligosaccharides. Milks with the highest ratios were produced primarily by Le^{a-b-} mothers; those with the lowest ratios were produced exclusively by Le^{a-b+} mothers ($p < 0.001$). Thus maternal genetic polymorphisms expressed as Lewis blood group types are expressed in milk as varied fucosyloligosaccharide ratios. The four infants who developed diarrhea associated with stable toxin of *Escherichia coli* were consuming milk with lower ratios (4.4 ± 0.8 [SE]) than the remaining infants (8.5 ± 0.8; $p < 0.001$). Furthermore, the 27 infants who developed moderate to severe diarrhea of any cause were consuming milk with lower ratios (6.1 ± 0.9) than the 26 who remained healthy (10.5 ± 1.9; $p = 0.042$). Thus, milk with higher 2-linked to non-2-linked fucosyloligosaccharide ratios affords greater protection against infant diarrhea. We conclude that specific oligosaccharides constitute a major element of an innate immune system of human milk.

Key words: diarrhea/fucosyltransferases/Lewis blood groups/secretor/stable toxin of *E. coli*

Introduction

The oligosaccharides, found in concentrations of up to 12 g/L, are the third largest constituent of human milk. Human milk oligosaccharides typically contain a lactose moiety at the reducing end and a fucose at the nonreducing end. Oligosaccharides of type 1 structure may have fucosyl α1,4 linked to *N*-acetylglucosamine, whereas those of type 2 structure may have fucosyl α1,3 linked to *N*-acetylglucosamine or glucose; either type may contain fucosyl α1,2 linked to galactose. The addition of fucose to an oligosaccharide by an α1,2 linkage is catalyzed primarily by a fucosyltransferase produced by the secretor gene, *Se* (*FUT2*); the addition of fucose by an α1,3 or α1,4 linkage is catalyzed by fucosyltransferases produced by the Lewis gene, *Le* (*FUT3*) or other α1,3 transferase genes (*FUT4*, *5*, *6*, *7*, and *9*) of this family (Oriol *et al*., 1999). Figure 1 describes our proposed synthesis of milk oligosaccharides by pathways defined by these enzymes.

Variation in the activities of the 2- and 3/4-fucosyltransferases can result from inactive or partially active genetic polymorphisms. Such variation can produce milk phenotypes that vary in relative quantities of specific fucosyloligosaccharides. For example, the nonsecretor phenotype results from inactive polymorphisms of the secretor gene in human populations. Women who are nonsecretors do not express measurable 2-linked fucosyloligosaccharides in their milk or other bodily fluids. In most populations found in or derived from Europe, the prevalence of nonsecretors is approximately 20%, whereas in other populations it can be much lower. In the mestizo population of Mexico, a population of mixed but primarily indigenous ancestry, the prevalence of nonsecretors is approximately 1% or lower (Erney *et al*., 2000; Henry *et al*., 1995). However, the expression of milk fucosyloligosaccharides varies even among secretors (Chaturvedi *et al*., 2001; Erney *et al*., 2000; Thurl *et al*., 1997; Viverge *et al*., 1990).

Polymorphisms of the secretor and Lewis genes also control expression of the Lewis blood group type (Erney *et al*., 2000; Henry *et al*., 1995). The different histo-blood group types in humans are associated with heterogeneous expression of glycoconjugates in erythrocytes and other tissues. Varying expression of these glycoconjugates can be associated with differential risk of infectious diseases (Blackwell *et al*., 1986a,b,c; Kallenius *et al*., 1980; Lomberg *et al*., 1983; Newburg, 1997; Newburg *et al*., 1993). For example, differential expression of Lewis or ABO histo-blood group types can be associated with varying risks of infection and diseases of the gastrointestinal tract (Glass *et al*., 1985; Huang *et al*., 2003; Ikehara *et al*., 2001; Ruiz-Palacios *et al*., 2003), presumably through differential expression of cell surface

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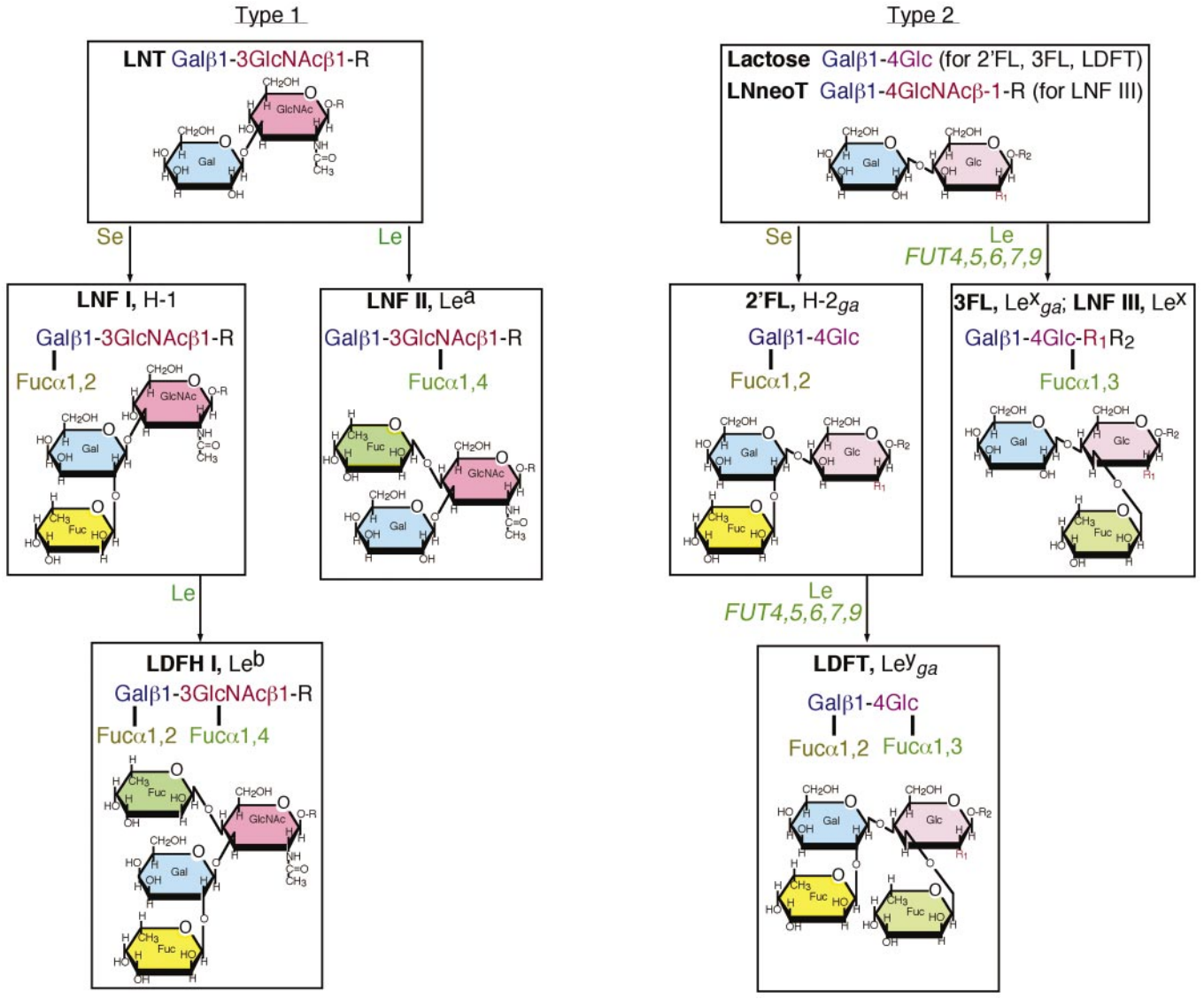

Fig. 1. Proposed synthetic pathways of the principal fucosyloligosaccharides of human milk. The principal Type 1 fucosyloligosaccharides are synthesized from LNT, that is, Galβ1,3GlcNAc on the terminal end of lactose (-R). LNT can be the substrate for fucosyltransferase III (Le), a product of the *FUT3* (i.e., Lewis) gene, to produce LNF-II, which is the Le^a antigen. Alternatively, LNT can be acted on by fucosyltransferase II (Se), a product of the *FUT2* (i.e., secretor) gene, to produce LNF-I, which is the H-1 antigen. This LNF-I can then be acted on by fucosyltransferase III (Le) to produce LDFH-I, the Le^b antigen. The principal Type 2 fucosyloligosaccharides are synthesized mainly from lactose, but also from lacto-*N*-neotetraose, that is, Galβ1,4GlcNAc on a lactose terminus. Synthesis from lactose results in glucose analogs (*ga*) of these Lewis antigens, where $-R_1$ is –OH and $-R_2$ is –H; true Lewis structures, such as LNF-III, have an $-R_1$ of *N*-acetyl and an $-R_2$ of lactose or lactosamine. These precursors can be acted on by fucosyltransferase III (Le), or perhaps for this type 2 pathway, fucosyltransferases IV, V, VI, VII, or IX (all of which catalyze the synthesis of 3-fucosyl linkages) to produce LNF-III, a Le^x antigen, or 3-FL, the glucose analog of Le^x, Le^x_{ga}. Alternatively, these type 2 substrates can be acted on by fucosyltransferase II (Se) to produce 2-fucosyllactose (2′-FL), the glucose analog of H-2 antigen. 2′-FL can be acted on by fucosyltransferase III (Le) or perhaps fucosyltransferases IV, V, VI, VII, or IX to form LDFT, the glucose analog of Le^y antigen. Note that blood group A and B epitopes, synthesized from the blood type O epitopes H-1 and H-2 in other tissues, are not found in appreciable quantities in human milk. However, each of the major neutral Lewis antigens are represented as a major fucosyloligosaccharide in human milk.

glycoconjugates that are used as receptors for pathogens of the intestinal mucosa.

The many human milk oligosaccharides and their related glycoconjugates include some that are bioactive. Milk glycoconjugates that have structural homology to the glycan moieties of intestinal mucosal cell surface may act as competitive inhibitors of pathogen binding to their glycoconjugate receptors. Examples include human milk oligosaccharides containing α1,2-linked fucose that inhibit the stable toxin-producing *Escherichia coli in vitro* and its toxin-induced secretory diarrhea *in vitro* and *in vivo* (Crane *et al.*, 1994; Newburg *et al.*, 1990). Glycoconjugates found in

human milk also inhibit binding by campylobacter *in vitro*, *ex vivo*, and *in vivo* (Cervantes *et al*., 1995; Ruiz-Palacios *et al*., 2003), and inhibit binding by caliciviruses *in vitro* (Huang *et al*., 2003; Jiang *et al*., forthcoming; Marionneau *et al*., 2002). Thus specific fucosyloligosaccharides of human milk have been observed to inhibit specific pathogens, but the clinical relevance of their presence in human milk, that is, the relationship between these milk oligosaccharides and risk of disease in a breastfeeding population, had not been tested.

We previously reported highly heterogeneous expression of milk fucosyloligosaccharides among individual mothers. Milk fucosyloligosaccharide expression also changes qualitatively and quantitatively over the course of lactation (Chaturvedi *et al*., 2001). The ratio of the α1,2-linked fucosyloligosaccharides to those that contain only α1,3- and α1,4-linked fucose declines exponentially over the first year of lactation. This pattern suggests coordinated reciprocal control of the synthesis of fucosyloligosaccharides, although the biological basis of this change over lactation is unknown. Individual lactating mothers exhibit similar exponential changes in this oligosaccharide ratio over the course of lactation, but at any given stage of lactation absolute values of this ratio often differ among mothers. This heterogeneous expression of human milk fucosyloligosaccharides among individual lactating mothers provides an opportunity to study both the basis of this variation and the role of milk oligosaccharides in protecting infants from enteric pathogens.

The current study was designed to test the hypothesis that fucosyloligosaccharides are part of a milk-borne innate immune system; if true, this necessitates that (1) fucosyloligosaccharide expression in human milk be constituative and, therefore, depend on maternal genotype; and (2) fucosyloligosacchride expression in milk be relevant to protection of breastfed infants against infectious disease. To test whether expression of human milk fucosyloligosaccharides is innate, we examined covariation of milk fucosyloligosaccharides of mothers in relation to their Lewis blood group type, which implies control by the same fucosyltransferase genes. To test whether fucosyloligosaccharide variation in maternal milk is clinically relevant, we examined the effect of predominance of 2-linked over non-2-linked fucosyloligosaccharides in milk in relation to protection of breastfed infants against diarrhea associated with stable toxin (ST)-*E. coli* and diarrhea in general.

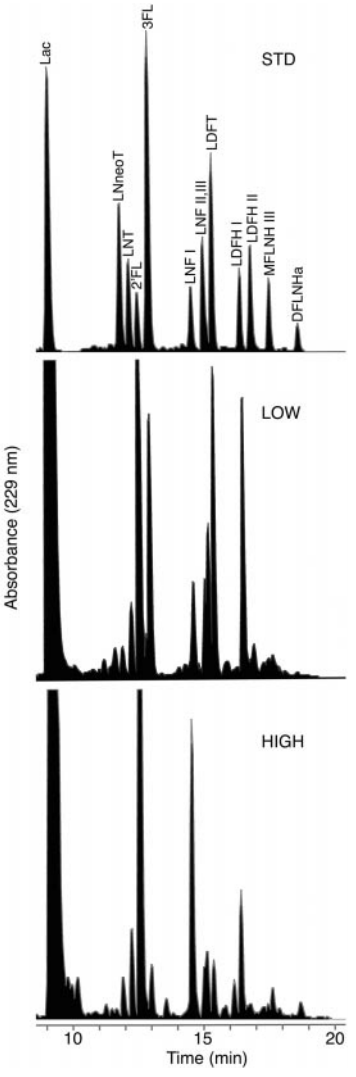

Fig. 2. Detection of the principal oligosaccharides of human milk by HPLC. Human milk samples (1 mL) were defatted by centrifugation; ethanol precipitation (67%) was used to remove the bulk of protein and lactose, yielding the crude oligosaccharide fraction. The oligosaccharides were perbenzoylated with benzoyl anhydride, purified on a reversed-phase column, resolved by reversed-phase HPLC, and detected at 229 nm. LNF-II and LNF-III coelute. STD shows a mixture of standards for lactose and the major oligosaccharides in human milk. LOW shows the profile of the oligosaccharides in a milk sample that had low relative amounts of 2-linked oligosaccharides. Note the relative amounts of 2′-FL to 3-FL and of the 2-linked LNF-I to LNF-II, -III. HIGH shows the profile of the milk oligosaccharides from a different mother that has high relative amounts of 2-linked oligosaccharides. Again note the relative amounts of 2′-FL to 3-FL and of LNF-I to LNF-II, -III.

Results

Oligosaccharide profiles

Analysis of the oligosaccharide content of 93 individual milk samples was performed by high-performance liquid chromatography (HPLC); representative traces of the output are shown in Figure 2. The identity of each peak was determined by mass spectrometry and was confirmed each day by coelution with authentic standards; oligosaccharide samples were diluted such that the peak areas of interest were within the linear range for quantification. Each of the major milk oligosaccharide peaks was fully resolved except for lacto-*N*-fucopentaose (LNF)-II and LNF-III. This peak area was designated LNF-II in our analyses, because this was the major component of the peak and was the only measure that contained the Le^a moiety.

Each of the major milk oligosaccharide peaks corresponds to a Lewis structure shown in Figure 1. The concentrations of each of these eight major oligosaccharides in the milk samples is given in Table I. All 93 donors had 2-linked fucosyloligosaccharides in their milk and were thus secretors, consistent with the known low prevalence of

Table I. Oligosaccharides in human milk (weeks 1–5)

Milk oligosaccharide	Concentration (mmol/L)	% total oligosaccharides
LNT (Type 1)	1.27 ± 0.79	10.1 ± 4.6
LNF-I (H-1)	3.21 ± 1.75	25.1 ± 9.9
LNF-II and -III (Le^a)	1.15 ± 0.80	8.9 ± 3.8
LDFH-I (Le^b)	1.26 ± 1.00	9.5 ± 5.7
LNneoT (Type 2)	0.42 ± 0.26	3.3 ± 1.8
2′-FL (H-2_{ga})	3.85 ± 1.04	33.7 ± 10.4
3-FL (Le^x_{ga})	0.58 ± 0.96	4.3 ± 4.6
LDFT (Le^y_{ga})	0.70 ± 0.72	5.1 ± 3.4

$n = 93$; mean ± SD; *ga* = glucose analog. The peak for LNF-II also included LNF-III, a Le^x homolog.

Table II. Expected Hardy-Weinberg distribution of *Se* (*FUT2*) and *Le* (*FUT3*) genes

Genotype	*sese*	*Sese*	*SeSe*	Total
lele	0.0025	0.045	0.2025	0.25
Lele	0.005	0.09	0.405	0.50
LeLe	0.0025	0.045	0.2025	0.25
Total	0.01	0.18	0.81	

Secretor gene frequency: $se = 0.1$, $Se = 0.9$; Lewis gene frequency: $le = 0.5$, $Le = 0.5$.

nonsecretors in this population relative to a population of European descent (Erney *et al.*, 2000). Concentrations of type 1 and type 2 fucosylated oligosaccharides were similar. The most commonly occurring oligosaccharides were 2′-fucosyllactose (FL) (H-type 2) (3.8 mmol/L, 34% of total) and LNF-I (H-type 1) (3.2 mmol/L, 25% of total), followed by lacto-*N*-difucohexaose (LDFH-I) (Le^b), LNF-II (Le^a) and -III (Le^x), lactodifucotetraose (LDFT) (Le^y), and 3-FL (Le^x). LNT (type 1 precursor) was found in greater concentration (1.3 mmol/L) than LNneoT (type 2 precursor) (0.4 mmol/L). The variation for these measures was much greater than the variation intrinsic to the analytical method, suggesting large biological variation among different mothers in the milks they produced.

In erythrocytes and other tissues, as in milk, fucosylated glycan moieties are thought to be controlled primarily by two fucosyltransferase families: The 2-fucosyltransferase family consists of FucT-I (i.e., H), the product of the *FUT1* (*H*) gene, and FucT-II (i.e., Se), the product of the *FUT2* (secretor [*Se*]) gene. The 3/4-fucosyltransferase family consists of FucT-III (i.e., Le), the product of the *FUT3* (Lewis [*Le*]) gene (Thurl *et al.*, 1997; Viverge *et al.*, 1985), and FucT-IV, -V, -VI, -VII, and -IX, products of *FUT4*, *FUT5*, *FUT6*, *FUT7*, and *FUT9* genes, respectively (Henry *et al.*, 1995; Oriol *et al.*, 1999). Adapting this scheme to the synthesis of Lewis epitopes in milk oligosaccharides (Figure 1), these transferases would be expected to synthesize both type 1 and type 2 fucosyloligosaccharides. The core type 1 structure, lacto-*N*-tetraose (LNT), is a terminal Galβ1,3GlcNAc on lactose. Lactose is the core for the most abundant type 2 structures in milk (2′-FL, 3-FL, and LDFT), whereas lacto-*N*-neotetraose, Galβ1,4GlcNAc on a lactose terminus, is the core for LNF-III.

In other tissues, Lewis structural moieties are based on a lactosamine backbone (Gal-GlcNAc); however, the most prevalent type 2 fucosyloligosaccharides in human milk are synthesized from lactose (Gal-Glc) and therefore are defined as the glucose analogs to the type 2 Lewis structures. For both type 1 and type 2 pathways, the 2-fucosyltransferase and the 3- and 4-fucosyltransferases compete for the same substrates. Thus the relative activities of these fucosyltransferases are reflected in the combined content of 2-linked fucosyloligosaccharides relative to the combined content of fucosyloligosaccharides whose fucose is not 2-linked. Differences in these fucosyltransferase activities can be a result of differences in the expression of their respective genes; thus distinct genotypes could result in characteristically distinct patterns of oligosaccharide expression in milk. A single, sensitive phenotypic biomarker of the relative activities of these fucosyltransferases may be the ratio of the 2-linked to non-2-linked fucosyloligosaccharides.

Lewis blood group type

The bloods of the mothers in our cohort were typed for Lewis blood group by hemagglutination of the erythrocytes. Differences in maternal blood group type reflect expression of polymorphisms in maternal Lewis and secretor genotypes. The Lewis blood group distribution in study mothers was as follows: 67 were Le^{a-b+}, 24 were Le^{a-b-}, and the serologic classification for 2 mothers was Le^{a+b-}. However, because the milk of the two Le^{a+b-} mothers contained 2-linked fucosylated oligosaccharide, a finding inconsistent with being an obligate nonsecretor, the discrepancy between milk and blood group phenotypes was resolved by classifying the blood group as indeterminant and excluding these two mothers when calculating the distribution of Lewis blood group phenotypes.

Red cells adsorb fucosylated glycolipids (primarily type 1) from serum and therefore exhibit serological phenotypes that reflect the genotypes shown in Table II: the phenotype Le^{a-b-} is a manifestation of the lele genotype (i.e., homozygous recessive for the Lewis gene); Le^{a-b-} individuals express much less 3-linked fucose than the Le^{a-b+} phenotype individuals. The phenotype Le^{a-b+} is a manifestation of the four genotypes that contain at least one dominant Lewis gene and at least one dominant secretor gene. In Le^{a-b+} individuals, the Lewis fucosyltransferase (Le, or FucT-III) utilizes and depletes 2-linked fucosylated structures to produce the combined 2-, 3-, and 4-linked fucosylated structures, for example, Le^b for type 1 and Le^y for type 2; thus the Le^{a-b+} erythrocyte phenotype is negative for Le^a but positive for Le^b antigens.

Table III (Observed) contains the distribution of Lewis blood group phenotypes observed in our population, and Table II contains the distribution of genotypes inferred from the Hardy-Weinberg analysis of these observed

phenotypes and concordant phenotype data on this population reported by Erney *et al.* (2001). These data suggest that the prevalence of the nonsecretor (*se*) and secretor (*Se*) alleles may have a frequency of approximately $se = 0.1$, $Se = 0.9$, whereas the Lewis allele frequencies may be approximately equal ($le = 0.5$, $Le = 0.5$). This analysis predicts a distribution of Lewis phenotypes in this population (Table III, Expected) that almost exactly matches our observed values (Table III, Observed). Assuming a common genetic basis for expression of Lewis blood group in erythrocytes and expression of oligosaccharides in milk, the predicted genotype frequencies calculated in Table II were used to deduce a relationship between Lewis and secretor genes and milk oligosaccharide phenotypes.

Table III. Frequency of Lewis phenotypes

Lewis blood group type	Observed %	Expected %
a−b−	26	25
a−b+	74	74
a+b−	0	1

In Figure 3 the milk oligosaccharide distributions are shown in relation to maternal Lewis blood group phenotypes. Consistent with expectation based on erythrocyte phenotype, Le^{a-b-} mothers expressed less of the 3- and 4-linked fucose-containing oligosaccharides (LNF-II [Le^a], LDFH-I [Le^b], 3-FL [Le^x], and LDFT [Le^y]) in their milk when compared with Le^{a-b+} mothers. Also as expected, the 24 Le^{a-b-} mothers tended to have higher concentrations of oligosaccharides containing only α1,2-linkages (H antigens) when compared with Le^{a-b+} mothers.

The Lewis synthetic pathway (Figure 1) describes divergent pathways between the 2-linked fucosyloligosaccharides and fucosyloligosaccharides that contain only 3- and 4-linkages, which are thought to compete for common substrates. This reciprocal relationship might be best described in a single measure as the ratio of the sum of the 2-linked oligosaccharides (LNF-I [H-1], 2′-FL [H-2], LDFH-I [Le^b], and LDFT [Le^y]), whose synthetic pathway is initiated by the secretor gene product, to the sum of those fucosyloligosaccharides containing only 3- and 4- linkages (LNF-II [Le^a] and 3-FL [Le^x]), whose synthesis is initiated by the Lewis gene product. Consistent with expectation, Figure 4 demonstrates that this fucosyloligosaccharide ratio is

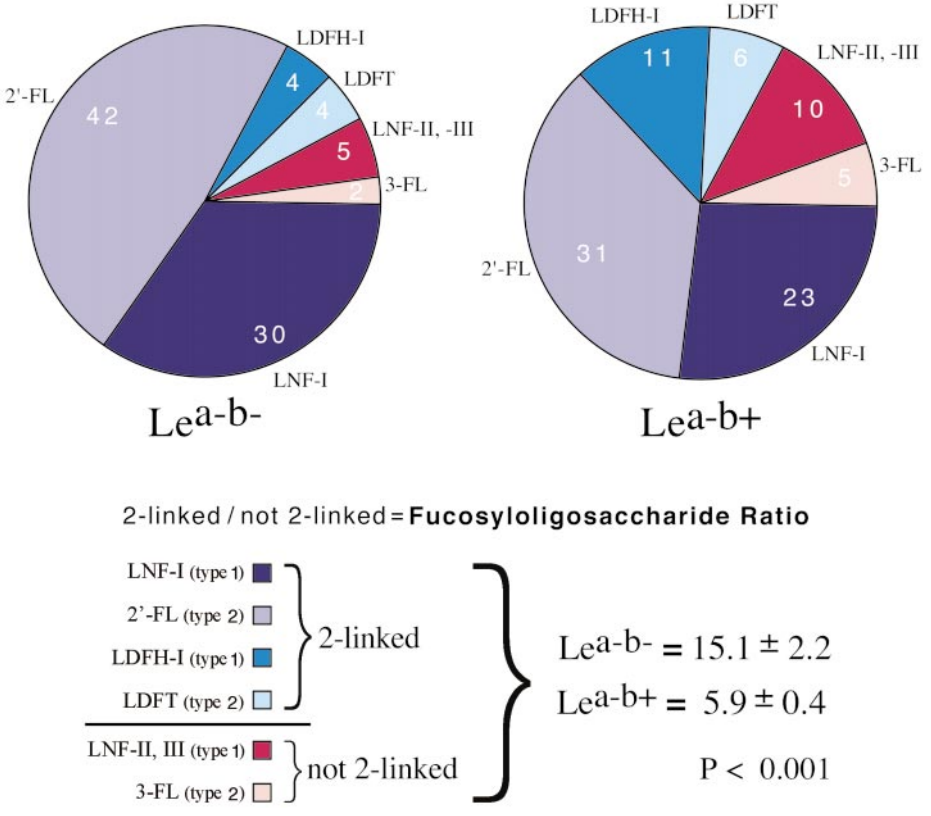

Fig. 3. The distribution of fucosyloligosaccharides in milk of mothers of differing Lewis blood group types. Serologic blood typing was used to assign the mothers to different Lewis blood group types. Two of the 93 mothers were of indeterminate types and were not included in this analysis. The content of the individual oligosaccharides as a percentage of the total milk oligosaccharides measured is shown for each type of mother. The standard errors of the percentage of each oligosaccharide for the milk of Le^{a-b+} mothers were all equal to or less than 1; for the Le^{a-b-} milks, they were equal to or less than 2. The differences in content of each of the oligosaccharides in milk of mothers of the two Lewis blood group types were tested by analysis of variance and Student's *t*-test, and were significant for each of the oligosaccharides: *p* value < 0.03 for LDFT, < 0.003 for LNF-I, and < 0.001 for all others. This difference in distribution of milk oligosaccharides in each individual of these two populations could be summarized as the ratio of 2-linked fucosyloligosaccharides to other fucosyloligosaccharides in a milk sample, that is (LNF-I + 2′-FL + LDFH-I + LDFT) divided by (LNF-II, -III + 3-FL). This ratio was significantly higher in milk from mothers of the Le^{a-b-} Lewis blood group type than Le^{a-b+} mothers.

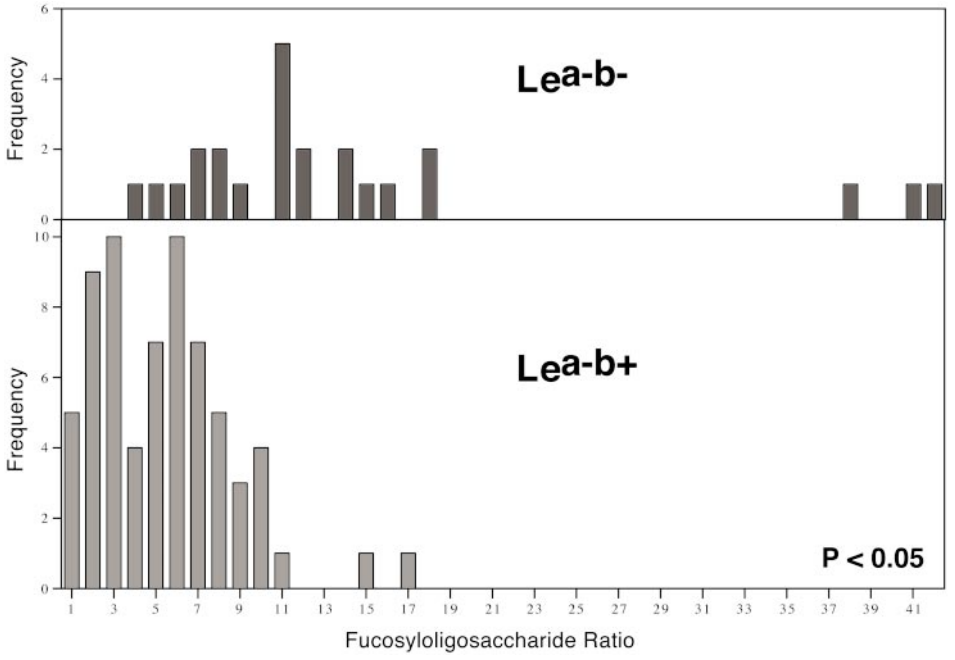

Fig. 4. Frequency distribution of the fucosyloligosaccharide ratio values of milk samples from the 67 Le^{a-b+} and the 24 Le^{a-b-} mothers. The distributions are significantly ($p < 0.05$) different by chi-square analysis.

significantly higher in milk produced by Le^{a-b-} mothers (15.1 ± 2.2) than in milk produced by Le^{a-b+} mothers (5.9 ± 0.4, $p < 0.001$). Of potential interaction terms or confounders that we considered, none was significant. The higher value for the fucosyloligosaccharide ratio in the milk of Le^{a-b-} mothers reflects a greater amount of 2-linked oligosaccharides and a lesser amount of the non-2-linked fucosyloligosaccharides when compared with the milk Le^{a-b+} mothers. This greater amount of 2-linked fucosylated oligosaccharides is made up of LNF-I and 2′-FL (H-1 and H-2, respectively) plus the total of LDFH-I (Le^b) and LDFT (Le^y). However, on their synthetic pathway LDFH-I (Le^b) and LDFT (Le^y) are synthesized from LNF-I (H-1) and 2′-FL (H-2) by the same fucosyltransferase enzymes that synthesize Le^a and Le^x. LNF-I (H-1) and 2′-FL (H-2), the dominant oligosaccharides of human milk, are found in greater amounts for Le^{a-b-} mothers. Therefore, despite the decrease in total LDFH-I (Le^b) and LDFT (Le^y), the total amount of 2-linked fucosylated oligosaccharides is higher in the milk of the Le^{a-b-} mother. Thus specific milk oligosaccharides make a disproportionate contribution to the fucosyloligosaccharide ratio values. The distribution of fucosyloligosaccharide ratios is shown in Figure 4 in relation to maternal Lewis blood group type. The milk fucosyloliogosaccharide ratio values for the Le^{a-b-} mothers range from 4 to 42, a significantly different distribution from the Le^{a-b+} mothers, all of whose milk fucosyloligosaccharide ratio values range from 1 to 18.

Relationship of milk fucosyloligosaccharide ratios to diarrhea in nursing infants

An oligosaccharide from human milk that contains 2-linked fucose is able to inhibit ST *in vitro* and *in vivo*. If the enzymes (i.e., secretor and Lewis gene products) that control the synthesis of the 2-linked fucosyloligosaccharides early in lactation likewise control the synthesis of the particular 2-linked fucosyloligosaccharide structure that protects against ST, one would predict a positive relationship between the ratios of 2- to 3/4-linked oligosaccharides and protection against ST-associated diarrhea in breastfeeding infants. Figure 5 demonstrates that the mean fucosyloligosaccharide ratio was significantly lower in milk consumed by infants with diarrhea due to ST-producing *E. coli* (4.4 ± 0.8 [SE]) than in the milk consumed by either infants asymptomatically infected with ST-producing *E. coli* (8.4 ± 1.0; $p = 0.04$) or uninfected control infants (8.6 ± 1.1; $p = 0.04$). Thus even in this population of mothers who are secretors, and who all produce 2-linked fucosyloligosaccharides, lower relative amounts of these 2-linked oligosaccharides were associated with increased risk of ST-associated diarrhea.

ST-*E. coli* is only one of many pathogens associated with diarrhea in this population; several other common enteric pathogens, for example, campylobacter (Ruiz-Palacios *et al.*, 2003) and caliciviruses (Huang *et al.*, 2003), are known to use 2-linked fucosylated glycoconjugates as receptors. Thus we analyzed the association between the fucosyloligosaccharide ratio in maternal milk and infant protection against diarrhea from all causes. We classified infants according to their history of diarrhea while they were breastfed: 27 (29%) infants had at least one case of moderate to severe diarrhea, 40 (43%) infants experienced at least one case of mild diarrhea but no cases of moderate to severe diarrhea while they were breastfed, and 26 (28%) infants never experienced diarrhea while they were breastfed. The mean fucosyloligosaccharide ratios of these groups are shown in Figure 6. The ratios in maternal milk

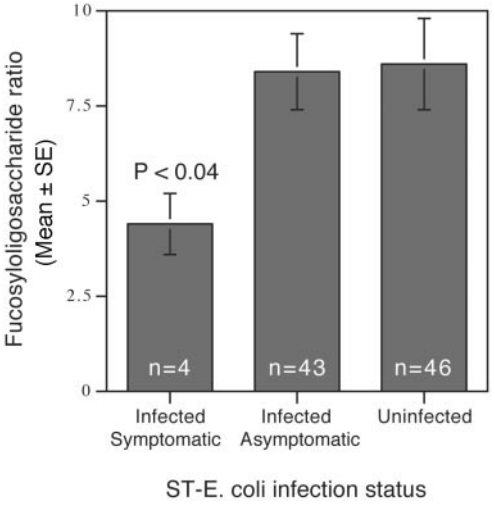

Fig. 5. Relationship between fucosylated oligosaccharide ratios and diarrheal symptoms among ST-*E. coli*–infected breastfeeding infants. The 93 breastfeeding mother–infant pairs who were followed prospectively were divided into three groups: infants who were infected with ST-*E. coli* and concurrently displayed symptoms of diarrhea (infected symptomatic), infants who were infected with ST-*E. coli* and did not have diarrhea (infected asymptomatic), and infants who were not infected with ST-*E. coli* (uninfected) while breastfeeding. The milk fed to infected symptomatic children had significantly lower fucosyloligosaccharide ratios than the milk of either the asymptomatic or noninfected groups.

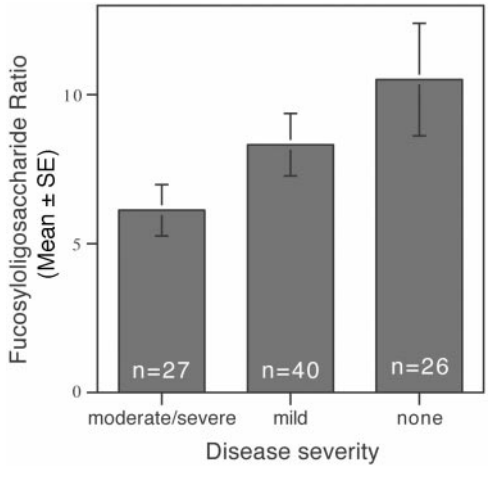

Fig. 6. Relationship between fucosylated oligosaccharide ratios and clinical symptoms of diarrhea due to all causes. The 93 mother–infant pairs who were followed prospectively were divided into three groups: 27 infants who had one or more cases of moderate to severe diarrhea while breastfeeding, 40 infants who had only mild diarrhea while breastfeeding, and 26 infants who never experienced diarrhea while breastfeeding. The milk consumed by infants who had one or more cases of moderate to severe diarrhea while breastfeeding had significantly lower fucosyloligosaccharide ratios than the infants who never experienced diarrhea while breastfeeding.

consumed by infants who had at least one case of moderate to severe diarrhea (6.1 ± 0.9 [SE]) was significantly lower than the ratios in milk consumed by infants who never experienced diarrhea while being breastfed (10.5 ± 1.9; $p = 0.04$). Of potential interaction terms or confounders that we considered, none was significant.

Discussion

Breastfed infants have a lower risk of diarrhea than infants fed milk substitutes. Heretofore this had been most commonly attributed to the antibody content of human milk. The present study finds that the content of 2-linked fucosyloligosaccharides in human milk is significantly associated with lower risk of diarrhea in breastfed infants, suggesting a major role for these oligosaccharides in immunity. We observed significant variation between mothers in the amounts and types of fucosyloligosaccharides expressed in their milk. This variation was summarized as the ratio of all major fucosyloligosaccharides containing 2-linked fucose to those containing no 2-linked fucose. A significant portion of this variation in milk was accounted for by differences in maternal Lewis blood group type.

The distribution of blood group types in a given population are manifestations of polymorphisms in genes whose products control glycoconjugate expression on the surface of red blood cells, generally at the nonreducing terminus of the glycan moiety. These same genetic polymorphisms are expressed in other tissues as variation in the production of cell surface carbohydrates, some of which are used as receptors by various pathogens. Associations have been reported previously between blood group type and susceptibility to specific diseases, especially those of mucosal surfaces. For example, individuals of O blood group type have greater susceptibility to cholera (Glass *et al.*, 1985) and certain strains of noroviruses (Huang *et al.*, 2003). P blood group types have been associated with different susceptibilities to hemolytic uremic syndrome (Newburg *et al.*, 1993). These genetic polymorphisms can also be expressed as variable production of glycoconjugates in secretions; the glycans of secreted glycoconjugates can bind to pathogens, inhibit binding by pathogens to their host, and thereby protect the host. For example, secretors, that is, those whose secretions contain glycoconjugates with α1,2-linked fucose moieties, appear to be less susceptible to urinary tract infections and infection by *Candida albicans* and *Haemophilus influenzae* than nonsecretors (Blackwell *et al.*, 1986a,b,c).

The distribution of Lewis blood group phenotypes in our study population differs from typical U.S. or European populations (Erney *et al.*, 2000) in that the Mexican mestizo population has a much lower prevalence of Lewis^{a+b-} (obligate nonsecretors) and a higher prevalence of Lewis^{a-b-}. We calculated a Hardy-Weinberg distribution of genotypes in this population (Table II), such that the distribution of Lewis and secretor genes predict erythrocyte phenotypes that match the observed phenotypes. The gene frequency of 0.9 for *Se*, the dominant secretor gene, fits well with the observation that most of these mothers are secretors. The frequency of 0.5 for *Le* (*FUT3*), the dominant Lewis gene, fits well with our finding a 3:1 ratio of Le^{a-b+} to Le^{a-b-}. This cohort appears to contain no true nonsecretors, based on the presence of measurable 2-linked fucosyloligosaccharides in the milk of each of the 93 nursing Mexican mothers.

We used this Hardy-Weinberg distribution of genotypes as a basis for interpreting the distribution of milk fucosyloligosaccharide phenotypes in this population. The milk of 67 Le^{a-b+} mothers contained significantly more α1,3- or α1,4-linked oligosaccharides, that is, LNF-II (Le^a) and 3-FL (Le^x), whereas the milk of the 24 Le^{a-b-} mothers contained more α1,2-linked oligosaccharides, that is, LNF-I (H-1) and 2′-FL (H-2). However, the amounts of oligosaccharides, even within each blood group type, had a wide distribution of patterns, with few discrete clustered groupings. Note that the milks of women with Le^{a-b-} blood group phenotypes had much lower concentrations of α1,4-linked oligosaccharides relative to the milk of women with Le^{a-b+} blood group phenotypes, consistent with our analysis; but our proposed synthetic scheme cannot account for the presence of any α1,4-linked structures in the milk of Le^{a-b-} mothers. The lack of a precise correspondence between the calculated Hardy-Weinberg distribution and observed milk oligosaccharide phenotypes could be due to several reasons. For example, there is the possibility that there are other missense mutations of the *FUT2* or *FUT3* genes not yet identified in this Mexican population that significantly lowered the α1,2-fucosyltransferase activity or α1,3/4-transferase activity. Other enzymes may make significant contribution to fucosyloligosaccharide synthesis in milk as compared to erythrocytes, such as FucT-I (H) as an alternative 2-fucosyltransferase, and FucT-IV, -V, -VI, -VII, or -IX as alternative 3-fucosyltransferases. The multimodal distribution of milk oligosaccharides, even within Lewis blood group types, suggests that several genotypes that express as one phenotype in erythrocytes could express as multiple phenotypes in milk. This may be due to a greater sensitivity of the HPLC of milk oligosaccharides relative to the serology used in Lewis blood group typing or may represent a true biological difference between glycan expression in milk oligosaccharides relative to that in erythrocyte glycolipids.

This continuum of oligosaccharide amounts and patterns in milk, which we presume contains the phenotypes for many possible genotypes, was expressed as a ratio of 2-linked to non-2-linked fucosyloligosaccharides in each milk sample. This ratio reflects the competition between the Lewis (FucT-III [Le]) and secretor (FucT-II [Se]) fucosyltransferases described in Figure 1 that underlie the differential phenotypic expression of Lewis moieties. Consistent with these observations, these oligosaccharide ratios distributed differently in milk of mothers of different Lewis blood group type. The highest fucosyloligosaccharide ratios were found primarily in the milks of Le^{a-b-} mothers, whereas milk with the lowest ratios was produced exclusively by Le^{a-b+} mothers. These ratios were also significantly related to protection of breastfeeding infants from disease.

Enterotoxigenic *E.coli* produce an ST that causes diarrhea. ST-induced diarrhea in mice can be inhibited by human milk (Cleary *et al*., 1983). This inhibitory activity is due to an α1,2-linked fucosylated oligosaccharide (Newburg *et al*., 1990). These fucosyloligosaccharides inhibit binding of stable toxin of *E. coli* to its host cell receptor *in vitro* (Newburg *et al*., 1995). If such inhibition of stable toxin were to also take place in the intestine of nursing infants, one would expect suppression of the symptoms of diarrhea in nursing children infected by ST-*E. coli* due to the presence of the specific α1,2-linked fucosyloligosaccharide inhibitor. If the same 2-fucosyltranferase that synthesizes the ST inhibitor also synthesizes the smaller 2-linked sugars measured in this study, there should be a direct relationship between a high ratio of 2-linked fucosyloligosaccharides and protection from ST-induced diarrhea. The four nursing infants in our cohort who were infected by ST-*E. coli* and developed diarrhea were consuming milk with significantly lower fucosylated oligosaccharide ratios than the milk consumed by those infants who were also infected but asymptomatic. This is consistent with α1,2-linked fucosyloligosaccharides of human milk playing a major role in providing significant protection to breastfed infants against ST of *E. coli*.

However, diarrhea associated with ST-*E. coli* accounts for less than 2% of total diarrhea cases in our population. Other more common enteric pathogens include campylobacter and noroviruses. Campylobacter binds to 2-linked fucosyl moieties of intestinal glycoconjugates, and its binding and infection are inhibited *in vitro* and *in vivo* by human milk fucosyloligosaccharides that have a 2-linked moiety, specifically the H-2 epitope (Ruiz-Palacios *et al*., 2003). Norovirus binding is also inhibited by fucosylated α1,2-linked glycans (Huang *et al*., 2003; Marionneau *et al*., 2002). Thus we investigated whether fucosylated oligosaccharides of human milk were also associated with protection of nursing infants against diarrhea due to pathogens other than ST. Infants who contracted one or more episodes of moderate to severe diarrhea while breastfeeding were consuming milk whose ratios were significantly lower than the milk consumed by infants who remained free of diarrheal symptoms while breastfeeding. Thus milk with higher 2-linked to non-2-linked fucosyloligosaccharide ratios affords greater protection against infant diarrhea due to all causes in this cohort. This suggests the presence of a family of oligosaccharides and/or glycoconjugates in milk, defined by their α1,2-linked fucose, that protect against several major enteric pathogens.

Our study population consisted only of secretors whose innate variation in the expression of 2-linked fucose is much less than that between secretors and nonsecretors. Nonetheless, we still found significant variation in the expression of α1,2-linked fucosyloligosaccharides in human milk, and found that this variation was significantly related to the incidence of diarrheal disease among breastfed infants. One might predict that protection against ST afforded by human milk might show even greater variation in a population that has greater differences in fucosyloligosaccharide expression, such as populations of European origin, which typically have 20% nonsecretors.

Some important enteric pathogens, for example, rotavirus, are inhibited by human milk oligosaccharides or glycoconjugates that are not fucosylated (Newburg *et al*., 1998; Smith *et al*., 1987). Thus the association we described in this study addresses only one possible set of enteric pathogens that may be inhibited by one family of milk oligosaccharides; other oligosaccharides that inhibit other pathogens are probable. We conclude that the family of α1,2-linked fucosylated oligosaccharides, probably in

conjunction with other families of oligosaccharides, constitute a powerful innate immune system of human milk.

A general hypothesis is that individual variation in oligosaccharide or glycoconjugate expression underlies varying susceptibility to different pathogens. The highly heterogeneous expression of glycoconjugates on cell surfaces would ensure that human populations are heterogeneous in their susceptibility to a given pathogen. Thus a major survival strategy for human populations periodically confronted with newly emergent, virulent, deadly pathogens would be that some proportion of the population is innately resistant by virtue of the heterogenous expression of glycoconjugates. Our data support a related hypothesis: oligosaccharides and glycoconjugates of milk protect infants from enteric pathogens because they contain epitopes homologous with intestinal receptors for pathogens, but expression of specific protective glycan epitopes vary among mothers of different genotype, providing a basis for selection of a population whose genotype provides the most protection in milk against pathogens. However, the hypothesis is confounded, as the genotypes of mothers with the highest innate protection to a given pathogen in their milk might also be expected to have infants with genotypes that have high probability of expression of the pathogen-binding epitope in their intestine, assuming both are mediated by the same genes. The expression of protective glycans in milk may be the stronger force for selection of traits in human populations. Milks containing the highest relative amount of epitopes with α1,2-linked fucose are most strongly associated with protection against ST, described herein, and campylobacter and some noroviruses, described elsewhere (Morrow *et al*., 2002). This phenomenon may help explain the prevalence of specific blood group types in regions where specific pathogens are endemic. For example, the low incidence of nonsecretors in the indigenous population of Mexico could be a consequence of the greater vulnerability of infants receiving milk containing less protective α1,2-linked glycans.

Despite the strong relationship, maternal blood group phenotype does not fully explain phenotype expression of fucosylated oligosaccharides in milk, but does support the conclusion that the expression of milk oligosaccharides is determined by maternal genetics. Genotyping of individual maternal fucosyltransferase genes is necessary to better understand variation in oligosaccharide expression in human milk. This study was limited to measurement of maternal phenotype and was not designed to provide genotype information for mother or infant, nor the infant's histo-blood group phenotype. Future studies are needed to clarify the genotype–phenotype relationship for breastfeeding mother–infant pairs and to relate this information to risk of diarrheal disease in children.

That notwithstanding, the strong relationship between the milk oligosaccharide phenotypes and the Lewis blood group phenotypes implies a common genetic basis of control: polymorphisms of the Lewis and secretor genes that underlie Lewis blood group phenotypes may also contribute to variation in the relative quantities of 2- to 3/4-linked fucosyloligosaccharides in human milk. Variation in expression of 2-linked fucosyloligosaccharides in human milk is significantly associated with variation in risk of disease in breastfed infants, supporting the conclusion that fucosylated oligosaccharides are a fundamental and potent mechanism of protection by human milk against infectious disease. Thus oligosaccharides may represent a significant component of an innate immune system of human milk whereby the lactating mother protects her nursing infant from environmental pathogens. The active moieties of these protective milk oligosaccharides may provide a basis for designing novel therapeutic agents for the prophylaxis and treatment of disease.

Materials and methods

Study design

From March 1988 through December 1991, a cohort of 316 mother–infant pairs was enrolled and monitored from birth to 2 years postpartum in San Pedro Martir, a transitional neighborhood of Mexico City (Morrow *et al*., 1992; Newburg *et al*., 1998; Velazquez *et al*., 1996). From the original cohort, 93 mother–infant pairs were selected for inclusion in this analysis based on: (1) having a history of breastfeeding, (2) having an adequate volume of a stored milk sample for oligosaccharide analysis, (3) sample collected between 1 and 5 weeks postpartum, (4) follow-up health data available for at least 2 weeks after sample acquisition, and (5) data available on the maternal blood group type. The mother–infant pairs included in this analysis were representative of the breastfeeding mother–infant pairs in the overall cohort. This research was approved by the institutional review boards of the Instituto Nacional de Ciencias Medicas y Nutricion in Mexico and Cincinnati Children's Hospital Medical Center.

Infant illness and feeding history were collected weekly. Stool samples were collected weekly with additional samples obtained whenever diarrhea occurred. Study outcomes included all diarrhea episodes, moderate to severe diarrhea using a standardized scoring system (Ruuska and Vesikari, 1990; Velazquez *et al*., 1996), and diarrhea due to ST-associated *E. coli*. Diarrhea episodes were defined as three or more watery stools within a 24-h period or loose to watery bowel movements that exceeded the child's usual daily stool frequency by two or more stools as determined by a study physician. Diarrhea due to ST-*E. coli* was defined as its detection in a stool sample collected during an episode of diarrhea. Testing for ST-*E. coli* was performed in the laboratory in Mexico using previously published methods (Lopez-Vidal *et al*., 1990). Maternal blood group typing was performed in the clinical laboratory in Mexico using postpartum blood samples.

Study population

The 93 mother–infant pairs in this study were monitored for up to 2 years postpartum. The analysis presented in this article was restricted to the duration of breastfeeding. The median duration of any breastfeeding was 9 months (range, 2 weeks to 24 months). Mothers' median age was 23 years; 22% completed a secondary or higher education; a median of five individuals lived in study households; and 33% of

infants were the first-born child. The demographic profile and infant feeding practices of this study population has been detailed elsewhere (Guerrero *et al*., 1999). The ABO distribution in study mothers was 18 (19%) A, 1 (1%) AB, 12 (13%) B, and 62 (67%) O blood group type. The Lewis blood group distribution in study mothers was 67 (72%) Le^{a-b+} and 24 (26%) Le^{a-b-}. The serologic classification for two of the mothers was Le^{a+b-}, which indicates obligate nonsecretors. However, because the milk of these two mothers contained 2-linked fucosylated oligosaccharide, inconsistent with being a nonsecretor, the discrepancy between milk and blood group phenotypes was resolved by classifying the blood group as indeterminant and excluding these two from further analysis related to Lewis blood group type.

Incidence of diarrhea

A total of 234 diarrhea episodes were identified in study children during 857 months of breastfeeding; the overall incidence of diarrhea was 28.8 cases per 100 child-months of follow-up ([number of episodes/months of follow-up] × 100). There were 77 (33%) episodes of moderate to severe diarrhea; the incidence of moderate to severe diarrhea was 9.3 cases per 100 child-months of follow-up. Over the course of breastfeeding, a median of two episodes of diarrhea occurred per child (range, 0–12 episodes), including a median of one moderate to severe diarrhea episode (range, 0–12 episodes). Symptomatic infection with ST-producing *E. coli* was identified in 4 (4%) of the 93 study children at some time during the follow-up period.

Milk samples were collected in the homes of study mothers by an experienced study nurse using an Egnell electric breast pump. Samples were transported on ice from the study household to the laboratory, where they were stored at −70°C. Milk samples were later transported to Boston, and oligosaccharide content was analyzed as described previously (Chaturvedi *et al*., 1997). Briefly, the human milk samples were centrifuged at 4,000 × *g* to separate the cream; the skimmed milk was made 67% with ethanol, stored overnight at 4°C, and recentrifuged; and the clear liquid was lyophilized to yield the crude oligosaccharide fraction. A 500-μg aliquot of the oligosaccharides from each sample was dried *in vacuo* for 4–6 h over phosphorus pentoxide. To each sample, 0.5 mL perbenzoylation reagent (benzoic anhydride [50 mg/mL] and 4-dimethylaminopyridine [25 mg/mL] in dry pyridine) was added (pyridine was predried over three sequential treatments of 4Å molecular sieves that had been freshly baked in an oven at 450°C overnight and cooled in a desiccator before use). After mixing, incubation was for 16 h at 37°C. Water (4.5 mL) was added to each sample, and the resulting solution was passed twice over a C-18 Bond-Elut column (3 mL, 0.5 mg; Varian, Sunnyvale, CA). The C-18 columns had previously been wetted with HPLC acetonitrile (5 mL) and equilibrated with 5 mL 1% pyridine in water and fitted onto a vacuum manifold with adequate vacuum to achieve a flow rate of 0.5 mL/min. After washing the column with 5 mL 10% pyridine and 5 mL HPLC water, the perbenzoylated oligosaccharides were eluted with 5 mL HPLC acetonitrile; the eluate was dried under N_2 and taken up in 100 μL HPLC acetonitrile.

The resulting perbenzoylated oligosaccharides were resolved by reversed-phase HPLC (C-8 column, 3 μ; 4.6 mm × 10 cm) at 1 mL/min with a 15-min linear gradient from acetonitrile:water (4:1) to 100% acetonitrile, with holding at the final condition for an additional 10 min. The perbenzoylated oligosaccharides were detected at 229 nm, and their peaks were integrated on a Macintosh computer with Rainin Dynamax software (Emeryville, CA). All of the major milk oligosaccharides produced a peak that was fully resolved and in the linear range of detection, except that LNF-II and LNF-III coelute in this system.

Statistical analysis

Incidence rates of diarrhea were calculated as the total number of cases per 100 child-months at risk (i.e., birth to the end of breastfeeding or termination from study). The major milk oligosaccharide Lewis antigen homologs were measured in individual milk samples as concentrations (mmol/L). These oligosaccharide values were then used to calculate a fucosyloligosaccharide ratio for each milk sample. The fucosyloligosaccharide ratio was defined as the ratio of 2-linked to non-2-linked fucosyloligosaccharides in the milk sample of each individual. We examined the association between maternal milk ratios in relation to maternal Lewis blood group type (i.e., Le^{a-b+} versus Le^{a-b-}); the association between infants who developed and did not develop ST-associated diarrhea; and the association between milk oligosaccharide ratios and diarrhea episodes classified into three categories by severity of diarrhea: infants who had moderate to severe, mild, and no episodes of diarrhea while breastfeeding. Analysis of variance, general linear model, two-sample *t*-tests, and median test were used where appropriate to evaluate these comparisons. Potential confounding and interaction effects were assessed by general linear model for any affects of maternal sociodemographic factors and of ABO blood group type on the relationship between milk oligosaccharides and risk of diarrhea and on the relationship between Lewis blood group type and oligosaccharide expression.

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Abbreviations

FL, fucosyllactose; FucT, fucosyltransferase; HPLC, high-performance liquid chromatography; LDFH, lacto-*N*-difucohexaose; LDFT, lactodifucotetraose; Le, Lewis; LNF, lacto-*N*-fucopentaose; LNT, lacto-*N*-tetraose; Se, secretor; ST, stable toxin.

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