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Receptor-Like Glycocompounds in Human Milk That Inhibit Classical and El Tor *Vibrio cholerae* Cell Adherence (Hemagglutination)

J. HOLMGREN,* A.-M. SVENNERHOLM, AND M. LINDBLAD
Department of Medical Microbiology, University of Göteborg, S-413 46 Göteborg, Sweden

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The two biotypes of *Vibrio cholerae* were found to have cell-associated hemagglutinins which differ with regard to binding to different species of erythrocytes and inhibition by monosaccharides. A total of 12 classical *V. cholerae* strains (Inaba or Ogawa) strongly agglutinated human erythrocytes in a reaction specifically inhibited by L-fucose, whereas 12 El Tor strains preferably agglutinated chicken erythrocytes, a reaction reversed by D-mannose or by higher concentrations of D-fructose, D-glucose, α-methyl-D-mannoside, or sucrose. Milk from Swedish women inhibited both of these adherence reactions, and the predominating inhibitory activity for each reaction resisted boiling, was destroyed by periodate treatment, and bound to a concanavalin A-Sepharose column, suggesting a carbohydrate structure. Further characterization indicated that the inhibitory activity for classical *V. cholerae* hemagglutination was distributed about equally on glycoprotein and free oligosaccharide, but was not present on glycolipid. The El Tor inhibiting activity, on the other hand, was almost exclusively of a high-molecular-weight glycoprotein nature. These results support our previous suggestion (Holmgren et al., Infect. Immun. 33:136–141, 1981) that human milk may contain receptor-like glycocompounds which can prevent bacterial adherence by competition with receptors on target cells.

Infection with enterotoxinogenic bacteria is an important cause of watery diarrheal disease, which may lead to dehydration and, in severe cases, death of patients. Colonization with adherence of bacteria to the intestinal epithelium and binding of secreted enterotoxin(s) to mucosal cells are obligatory pathogenic events in the enterotoxic enteropathies caused by these bacteria. Both bacterial adherence and enterotoxin action appear to depend on the presence of membrane receptors on the intestinal epithelial cells. Chemically, these receptors probably are glycoconjugates containing a receptor-specific oligosaccharide moiety linked to either a lipid or protein backbone which anchors the receptor in the cell membrane. (The term receptor is used in a loose sense simply to denote a specific cell surface binding site; the receptors for toxins are true receptors in that they partake in a transmembrane effect on cell metabolism, whereas the receptors involved in bacterial adherence are only known to be binding sites.)

In a previous study we found that samples of human milk had strong inhibitory activity for bacterial cell adherence, as well as for enterotoxin binding, as noted both for *Escherichia coli* possessing defined colonization factor antigens (adherence pili) and for *Vibrio cholerae* (9). The main inhibitory activity was separate from immunoglobulin, and we proposed that this activity might instead represent secreted structure analogs (oligosaccharides or glycoconjugates) of cell receptors for the particular bacterial adhesins and enterotoxins.

The aim of this study was to test this hypothesis by characterizing the factors in human milk that are responsible for the inhibition of adherence of *V. cholerae* cells to erythrocytes. *V. cholerae* is the prototype for those organisms which produce diarrheal disease by colonizing the small intestine and elaborating an enterotoxin, yet relatively little is known about its mode of adherence to cells, in contrast to the detailed knowledge that exists about the binding of cholera toxin to its cell receptors (7). However, *V. cholerae* cells have been found to cause agglutination of certain species of erythrocytes, and it has been suggested that the attachment of bacteria to erythrocytes mimicks the interactions that result in adherence of cholera vibrios to intestinal epithelium (1–3, 11; G. L. Bales and C. E. Lankford, Bacteriol. Proc., p. 118, 1961). At least two cell-associated hemagglutinins of *V. cholerae* have been described; one of these is

inhibited by L-fucose, and the other is inhibited by D-mannose (1, 5, 11). We have found that the fucose-sensitive hemagglutinin is associated with the classical biotype of *V. cholerae* and that the mannose-sensitive hemagglutinin is associated with the El Tor biotype. Both of these hemagglutinins are strongly inhibited by human milk, and our results indicate that an oligosaccharide(s) and a high-molecular-weight glycoprotein(s), probably containing fucose, are responsible for the hemagglutination inhibition (HAI) of classical *V. cholerae* strains and that a high-molecular-weight milk glycoprotein which presumably contains mannose mediates inhibition of El Tor cholera vibrio hemagglutination (HA).

(A preliminary account of the results was presented at the International Symposium on Enteric Infections, Brügge, Belgium, 7–9 September 1981.)

MATERIALS AND METHODS

Bacteria. A total of 24 strains belonging to *V. cholerae* O group 1 were used; 12 of these strains were of the classical biotype, and 12 strains were of the El Tor biotype. These strains and their serotypes (Inaba or Ogawa) are listed in Table 1. One classical strain (1451 Ogawa) and one El Tor strain (1836 Inaba) were used for studying inhibitory milk compounds; these strains were selected as adherence prototypes for the respective *V. cholerae* biotypes due to their high stable hemagglutinating capacities for different species of erythrocytes.

The bacteria were grown with shaking (200 rpm) in Trypticase soy broth without glucose at 37°C for 7 to 8 h; an overnight culture in Trypticase soy broth served as the inoculum. After storage at 4°C overnight, the bacterial cultures were centrifuged at 10,000 × *g* for 10 min, and the resulting sediments were suspended in a modified Krebs-Ringer solution (KRT buffer; pH 7.4) (10) to a final concentration of 2×10^{10} cells per ml, as adjusted spectrophotometrically. The bacteria were then kept chilled at 0 to 4°C until they were used, usually on the same day or at most 1 day later. These cultivation and handling procedures have been found to be suitable for the expression of good amounts of cell-associated hemagglutinins on both classical and El Tor cholera vibrios and to minimize the production of a soluble hemagglutinin(s) that may also be produced in cultures of either classical or El Tor cholera vibrios (3, 5; Svennerholm, Jonsson-Strömberg, and Holmgren, submitted for publication).

Erythrocytes. Human erythrocytes of blood groups O, A, and B were used, along with bovine, chicken, guinea pig, mouse, and sheep erythrocytes. The erythrocytes were collected in sodium citrate and were washed three times in KRT buffer before use in the HA experiments.

Carbohydrates. The following sugars obtained from Sigma Chemical Co., St. Louis, Mo., were used: D-fructose-6-phosphate, L-(−)-fucose, D-(+)-glucose, D-glucose-6-phosphate, D-mannose-6-phosphate, α-methyl-D-galactoside, and α-methyl-D-mannoside. In addition, we also used L-(+)-arabinose, D-(+)-galactose, D-(+)-mannose, and D-(−)-ribose from E. Merck AG, Darmstadt, West Germany, methyl-α-D-glucopyranoside and *N*-acetylglucosamine from Fluka AG, Buchs, Switzerland, D-fructose from Pfanstichl Laboratories, Inc., Waukegan, Ill., lactose from May & Baker Ltd., Dagenhem, England, and sucrose from Fisher Scientific Co., Fairlawn, N.J.

TABLE 1. Inhibition of classical and El Tor *Vibrio cholerae* HA by L-fucose and D-mannose, respectively

Biotype	Serotype	Strain	HA[a]
Classical	Ogawa	34[b]	FS, MR
		84[b]	FS, MR
		1451[b]	FS, MR
		19766[c]	FS, MR
	Inaba	1[b]	FS, MR
		35[b]	FS, MR
		48[b]	FS, MR
		86[b]	FS, MR
		57[b]	FS, MR
		V63P[d]	FS, MR
		1449[b]	FS, MR
		569B[b]	FS, MS
El Tor	Ogawa	1796[b]	MS, FR
		1800[b]	MS, FR
		1824[b]	MS, FR
		1850[b]	MS, FR
		T20859[d]	MS, FR
		T19218	MS, FR
		O17SR[e]	MS, FR
	Inaba	1836[b]	MS, FR
		1843[b]	MS, FR
		T19479[d]	MS, FR
		T20567[d]	MS, FR
		Phil 6973[f]	MS, FR

[a] HA by classical vibrios was examined with chicken erythrocytes, and HA by El Tor vibrios was examined with human O erythrocytes; 1 mg of L-fucose or D-mannose per ml was used for the HAI tests. FS, Fucose sensitive; MR, Mannose resistant; MS, mannose sensitive; FR, fucose resistant.

[b] From H. Smith, Philadelphia, Pa.

[c] From I. Huq, International Centre for Diarrheal Disease Research, Dacca, Bangladesh.

[d] From S. C. Pal, Calcutta, India.

[e] From W. Chaicumpa, Bangkok, Thailand.

[f] From Statens Seruminstitut, Copenhagen, Denmark.

Milk specimens. A pool of milk from Swedish mothers was used. The fresh milk was centrifuged at 15,000 × *g* for 30 min, and the clear middle layer was separated from fat and cells and frozen in portions at −30°C. For the lipid extraction experiments noncentrifuged pooled Swedish whole milk was used.

Fractionation of milk. (i) Ultrafiltration. The milk was separated into a high-molecular-weight fraction (fraction A [molecular weight, ≥10,000]) and two low-molecular-weight fractions (fraction B [molecular weight, 1,000 to 10,000] and fraction C [molecular weight, <1,000]) by ultrafiltration through Diaflo (Amicon Corp., Lexington, Mass.) membrane filters. Fraction A was produced by passing the milk through a type PM-10 membrane filter; the retentate was diluted with 10 volumes of KRT buffer (pH 7.4) (10)

TABLE 2. Inhibition of classical and El Tor *V. cholerae* HA by different sugars

Sugar	*V. cholerae* HA MIC (mg/ml)[a]	
	Classical	El Tor
D-Ribose	>25	NT[b]
L-Arabinose	>25	>25
D-Glucose	>25	0.08
D-Mannose	>25	0.02
D-Galactose	>25	25
D-Fructose	25	0.04
L-Fucose	0.012	>25
Sucrose	>25	0.17
Lactose	>25	25
D-Glucose phosphate	25	>25
D-Mannose phosphate	>25	>25
D-fructose phosphate	>25	25
α-Methyl-D-glucopyranoside	>25	2.5
α-Methyl-D-mannoside	>25	0.05
α-Methyl-D-galactoside	NT	>25
N-acetylglucosamine	>25	5

[a] Two classical strains (strains 1449 and 1451) and two El Tor strains (strains 1836 and T19479) were tested with human O and chicken erythrocytes, respectively. MIC, Minimal inhibitory concentration.

[b] NT, Not tested.

and refiltered through the membrane to wash out the material with a molecular weight of less than 10,000. The final retentate was concentrated 2.4-fold in relation to the applied milk. The material passing through the type PM-10 membrane was similarly filtered through a type UM-2 membrane filter, and the retentate was diluted with 10 volumes of KRT buffer and refiltered to eliminate material with a molecular weight of <1,000. The retentate (fraction B) was concentrated fivefold in relation to the applied milk, and fraction C, which passed through the filter was not diluted. All fractions were frozen in portions at −30°C until they were used.

(ii) Immunosorbent chromatography. Fraction A was filtered through an immunosorbent column containing anti-human immunoglobulin A α-chain-specific antibody and anti-human total immunoglobulin (Dakopatts, Copenhagen, Denmark) covalently coupled to Sepharose (9). After passage several times through the gel, nonbound material was washed out with KRT buffer, and specifically bound material was eluted with acid buffer (1 M acetate, 0.5 M NaCl, 0.5 M glycine, pH 3.2) as previously described (9); after dialysis against KRT buffer, the fractions were concentrated to the volume of milk applied.

(iii) Preparation of casein-free milk. Whole milk and fraction A were acidified with 0.1 M sodium acetate to pH 4.5. After incubation at room temperature for 30 min and centrifugation at 10,000 × *g* for 20 min, the resulting casein-free supernatants were neutralized and analyzed for HAI activity.

(iv) Ammonium sulfate precipitation. Milk fractions A and B were precipitated in the cold for 60 min with ammonium sulfate added to 40 and 100% saturation, respectively. The precipitates obtained by centrifugation at 10,000 × *g* for 2 min were dissolved in ~50 volumes of KRT buffer and then concentrated to the original volume on membrane filters as described above. The procedure was repeated once. The same dilution-concentration procedure was performed with the supernatants containing saturated ammonium sulfate.

(v) Periodate treatment. Sodium periodate was added to either whole milk or fraction A to a final concentration of 0.05 M. Then the sample was boiled for 10 min, incubated at 37°C for another 24 h, and kept at 4°C for 3 days. After centrifugation at 10,000 × *g* for 2 min, the resulting supernatants were diluted and concentrated several times with membrane filters to decrease the periodate concentration to less than 0.0001 M.

(vi) Binding to concanavalin A. Fractions A and B were applied in 1-ml volumes to columns containing 1.2 ml of concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden). After three passages of the milk fraction through the column, nonbound material was washed out with 5 ml of Tris buffer (0.02 M Tris, 0.15 NaCl, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$, pH 7.4). Specifically bound material was then eluted with 5 ml of 0.5 M α-methyl-D-glycopyranoside. The wash and eluate fractions were concentrated to 1 ml by ultrafiltration through type PM-10 (fraction A) and type UM-2 (fraction B) membranes, respectively. The eluate fractions were then diluted 10-fold in water and refiltered to eliminate the eluant, a procedure which was repeated four times. Since the capacity of the concanavalin A-Sepharose column was too low for fraction B, 1 ml of this material was also chromatographed as described above on a 10-fold-larger column.

(vii) Pronase digestion. Fraction A was subjected to pronase digestion by adding pronase (Sigma) to a final concentration of 2.3 mg/ml and incubating the mixture at 37°C for 96 h. After boiling for 10 min to stop the enzyme reaction, the mixture was centrifuged at 3,000 × *g* for 10 min. The supernatant was filtered through a type PM-10 membrane, and the retentate and the filtrate were analyzed for HA inhibitory activity.

(viii) Extraction of lipids and isolation of glycolipids. Lipids were isolated from pooled human milk by repeated extraction with 20 volumes of chloroform-methanol-water (4:8:3, vol/vol) (16), and a delipidized milk residue was collected as a sediment after centrifugation at 1,000 × *g* for 10 min. The lipid extracts were combined and separated into acidic and neutral lipids by ion-exchange chromatography (18), and these lipids were further purified by procedures described elsewhere (8) to give one fraction containing acidic glycolipids (gangliosides) and one fraction containing neutral glycosphingolipids.

HA and HAI tests. Bacterial HA was studied after adding 10-μl portions of a 3% erythrocyte suspension to 10-μl twofold serial dilutions of bacteria on microscopic glass slides. The HA pattern was recorded after the reactants were mixed at room temperature for 3 min. HA was graded as follows: no agglutination, weak agglutination, or strong agglutination. The highest bacterial dilution giving strong agglutination was defined as the HA titer. HAI tests were performed by mixing 10-μl portions of a bacterial preparation adjusted to contain 2 HA titer doses with 10-μl twofold serial dilutions of the inhibitory substance and then, after 1 min, adding 10-μl portions of an erythrocyte solution. After the reactants were mixed for another 3 min, the HA pattern was recorded; the highest dilution of the

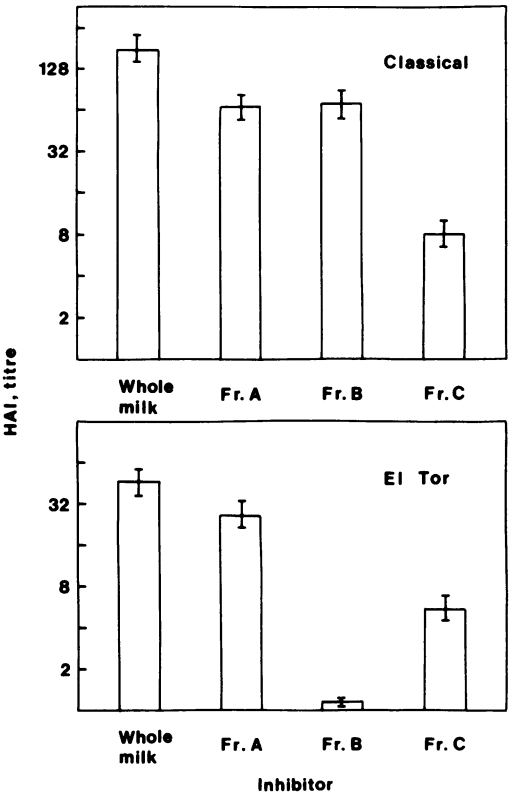

FIG. 1. Molecular weight distribution of HAI titers in human milk for classical and El Tor *V. cholerae*. The titers shown (mean ± standard error of the mean) are those obtained after correction for the higher concentration of fractions A and B compared with the concentration in the whole milk sample. Fr. A., Fraction A; Fr. B, fraction B; Fr. C, fraction C.

inhibitor giving no agglutination was determined. Each inhibitor sample was tested at least twice on two different occasions.

RESULTS

HA reactions by classical and El Tor *V. cholerae*. Two classical *V. cholerae* strains (1449 Inaba and 1451 Ogawa) and two El Tor strains (1836 Inaba and 1824 Ogawa) were tested for the ability to agglutinate various human and animal erythrocytes. Both classical strains, irrespective of serotype, gave strong HA with human O, A, and B erythrocytes, as well as with bovine, guinea pig, mouse, and sheep erythrocytes, and slightly weaker HA with chicken erythrocytes. Titrations showed that 10^8 classical *V. cholerae* cells per ml gave a HA reaction with human erythrocytes, whereas 10 to 20 times more bacteria were needed for HA of the different animal cells. In contrast, the two El Tor strains gave the strongest HA reactions with chicken, guinea pig, and mouse erythrocytes and weaker HA patterns with human, bovine, and sheep cells. On the basis of these results, human O erythrocytes were routinely used for assaying HA by classical *V. cholerae* and chicken erythrocytes were used for assaying HA by El Tor vibrios.

L-Fucose and D-mannose have been reported to inhibit HA reactions by *V. cholerae* (1, 11). These and a series of other mono- and disaccharides were tested for the ability to inhibit HA reactions by two selected classical strains and two El Tor strains. Only L-fucose gave inhibition of HA by the classical strains, whereas HA by the El Tor strains was inhibited by D-mannose, D-fructose, α-methyl-D-mannoside, D-glucose, and sucrose, but not by fucose (Table 2).

To determine whether these different inhibitory activities of certain sugars related to the *V. cholerae* biotype or serotype or both, 24 *V. cholerae* strains representing equal numbers of the classical and El Tor biotypes and representing both Inaba and Ogawa serotypes were tested for their HA reactions with human and chicken erythrocytes and for inihibition by L-fucose and D-mannose, respectively. Irrespective of serotype, all 12 El Tor strains gave HA reactions inhibited by mannose but not by fucose (Table 1). In contrast, all 12 classical *V. cholerae* strains gave HA reactions that were inhibited by fucose, and in all but one strain mannose gave no inhibition; the exception was strain 569B Inaba, which was inhibited by either fucose or mannose (Table 1).

HAI inhibitory compounds in human milk. (i) Fractionation according to molecular weight. A 500-ml pool of human milk was centrifuged at 15,000 × *g* for 30 min, which gave separation into a particulate sediment, a clear middle layer, and a fatty top layer. Titration experiments showed that all of the HAI activity of the whole milk both for classical *V. cholerae* tested with human erythrocytes and for El Tor vibrios tested with chicken erythrocytes was recovered in the clear middle layer; hence, this fraction was used for all further studies. The glass slide HAI titers based on five experiments with this fraction using 2 HA doses of classical and El Tor

TABLE 3. Separation of high-molecular-weight *V. cholerae* HA inhibitory activity of milk into a non-immunoglobulin fraction and an immunoglobulin fraction by immunosorbent chromatography[a]

Fraction	HAI titer	
	Classical	El Tor
Applied fraction A	32	32
Non-immunoglobulin fraction (wash)	16	16
Immunoglobulin fraction (eluate)	<2	<2

[a] Affinity chromatography was on a Sepharose–anti-human immunoglobulin column (see text).

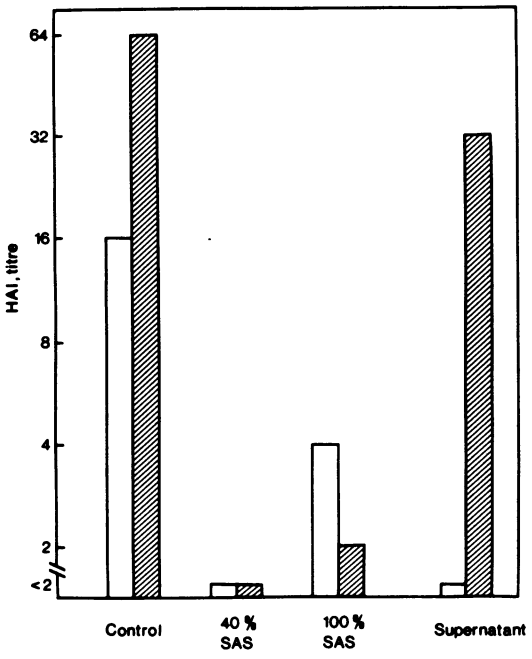

FIG. 2. Ammonium sulfate precipitabilities of HAI activities of human milk fractions A and B for classical *V. cholerae*. The HAI activity of fraction A precipitated with 100% saturated ammonium sulfate (SAS), but the HAI activity of fraction B did not. Open bars, Fraction A; cross-hatched bars, fraction B.

vibrios were 145 ± 18 and 64 ± 5 (mean ± standard error of the mean), respectively. The inhibitory concentrations of L-fucose for HA caused by classical vibrios and D-mannose for El Tor-mediated HA in the same experiments were 0.01 ± 0.002 and 0.01 ± 0.001 mg/ml, respectively, and in no instance did 10 mg of fucose per ml inhibit the El Tor HA reaction or 10 mg of mannose per ml inhibit the classical HA reaction.

The middle layer fraction of milk was then filtered through membranes with defined pore sizes in order to characterize the HAI activity for the two different *V. cholerae* biotypes with regard to approximative molecular weight. For classical *V. cholerae*, the HAI activity was distributed about equally in fraction A (molecular weight, >10,000) and fraction B (molecular weight, approximately 1,000 to 10,000), with only about 5% of the activity in fraction C (Fig. 1). In contrast, about 80% of the HAI activity for the El Tor vibrios was found in fraction A, less than 5% was in fraction B, and 15 to 20% was in fraction C (Fig. 1); in fivefold-concentrated fraction B a HAI titer of 4 to 8 could be demonstrated for El Tor vibrios (data not shown).

(ii) Immunoglobulins. Milk contains about 1 mg of immunoglobulin per ml, mainly immunoglobulin A (17). Even though the unchanged HAI titers after boiling did not support any major contribution of antibodies to the HAI activity of the pool of milk from Swedish women, we wanted to assess the possible role of immunoglobulin more directly (9). Therefore, high-molecular-weight fraction A, which contained all of the milk immunoglobulin (as assayed by immunodiffusion tests), was separated on an immunosorbent column to yield a non-immunoglobulin fraction and an immunoglobulin fraction, which were tested for HAI activity. The results showed that no detectable HAI activity was recovered in the immunoglobulin fraction (Table 3).

(iii) Heat stability. The heat stabilities of whole milk and separated fractions A through C were tested by heating samples at 100°C for 15 min and, after cooling, comparing the HAI titers with those of concurrently tested unheated samples. In no instance did the heat treatment cause any detectable decrease in the HAI titer.

(iv) Acid and salt precipitation. Caseins were removed from whole milk and fraction A by precipitation at pH 4.5, and the HAI activities of the casein-depleted neutralized samples were assayed. For both classical and El Tor vibrios the HAI titers were the same as in untreated samples, indicating that caseins were not responsible for the HAI effects (data not shown).

The precipitabilities of the HAI activities of fractions A and B by ammonium sulfate were also tested. At 40% ammonium sulfate saturation, no HAI activity was precipitated in either fraction. On the other hand, after precipitation at 100% ammonium sulfate saturation none of the HAI activity of fraction A remained in the supernatant, whereas the HAI activity of fraction B remained essentially unaffected (Fig. 2). Results were similar for classical and El Tor vibrios.

(v) Periodate sensitivity. The heat stability and ammonium sulfate precipitation data suggested that the main HAI activity of fraction A for both

TABLE 4. Binding of *V. cholerae* HA inhibitory activities to a concanavalin A-Sepharose column

Fraction	HAI titer	
	Classical	El Tor
Fraction A		
Fraction A, twofold concentrated	64–128	32–64
Unbound fraction	2–8	<2
Eluted fraction	2–8	8–64
Fraction B		
Fraction B, fivefold concentrated	256	4–8
Unbound fraction	64–128	<2–2
Eluted fraction	<2	4–8

TABLE 5. Effect of pronase digestion on high-molecular-weight HA inhibitory activities in human milk

Fraction	HAI titer	
	Classical	El Tor
Fraction A	32	32
Fraction A, pronase digested	16	32
Retentate of pronase-digested fraction A on PM-10 membrane	<2	<2
Filtrate of pronase-digested fraction A on PM-10 membrane	16	16

classical and El Tor *V. cholerae* was mediated by a glycoconjugate(s) and that the HAI activity of fraction B for classical *V. cholerae* was mediated by an oligosaccharide(s). The assumption that the milk components with HAI activity contained sugars was tested by assaying the sensitivity to periodate treatment. The results showed that the heat-stable HAI activities of whole milk and fractions A and B, against both classical and El Tor *V. cholerae* were completely destroyed by periodate treatment.

(vi) Binding to concanavalin A-Sepharose. Concentrated fractions A and B were applied to a concanavalin A-Sepharose column to determine how much of the HAI activity bound to and later could be eluted with sugar from the column. Table 4 shows that the El Tor HAI activities, both the strong activity in fraction A and the much weaker activity in fraction B, could be bound to and eluted from the lectin column (Table 4). Also, the HAI activity for classical *V. cholerae* in fraction A bound significantly to the concanavalin A column and could also be partially eluted from the concanavalin A-Sepharose. This contrasted with the HAI activity of fraction B for classical *V. cholerae*, which did not show any significant binding to and elution from the column (Table 4).

(vii) Glycoprotein or glycolipid high-molecular-weight activity. We performed experiments to examine whether the high-molecular-weight HAI activities were due to glycolipids or glycoproteins or both. Whole milk was lipid extracted, and acid glycolipids (gangliosides) and neutral glycosphingolipids were isolated. None of these glycolipid preparations had any detectable HAI activity for either classical or El Tor *V. cholerae*; on the other hand, the delipidized, denatured milk residue retained HAI activity for both types of vibrios (data not shown).

We then performed experiments to test the effect of pronase on the high-molecular-weight HAI activities in fraction A. The results showed that pronase digestion in the presence or absence of urea did not destroy any HAI activity for either classical or El Tor vibrios. However, the treatment dramatically changed the molecular weight of both of these activities, such that they completely passed through type PM-10 membranes (Table 5).

These results indicate that the HAI activities in fraction A for both classical and El Tor *V. cholerae* are of a glycoprotein nature rather than a glycolipid nature.

DISCUSSION

This study was undertaken to characterize the components in human milk that are responsible for the recently observed, strong inhibitory activity of milk for *V. cholerae* bacterial adherence to erythrocytes (9).

The HA assay is a convenient assay of bacterial attachment to erythrocytes, and there is some evidence that the cell-bound hemagglutinins on *V. cholerae* are indeed responsible for adherence of cholera vibrios to intestinal epithelium as well (2, 10). The nature of *V. cholerae* hemagglutinins is still incompletely known. Jones and Freter (11) found that L-fucose specifically inhibited HA, as well as adherence to intestinal epithelium by a *V. cholerae* strain, whereas D-mannose-sensitive *V. cholerae* HA has been described by other workers (1). A soluble *V. cholerae* hemagglutinin has also been described and purified (3; Svennerholm, Jonsson-Strömberg, and Holmgren, submitted for publication). Our data show that the cell-bound fucose- and mannose-sensitive hemagglutinins are closely associated with the *V. cholerae* biotype. A fucose-sensitive hemagglutinin which preferentially adheres to human erythrocytes was found on all classical strains tested, and a mannose-sensitive hemagglutinin with preference for chicken erythrocytes was found on all El Tor strains; the El Tor hemagglutinin could also be inhibited by D-fructose or D-glucose. The different effects of monosaccharides probably reflect the facts that L-fucose is a critical constituent of the receptor for classical *V. cholerae* and D-mannose is a component of the El Tor vibrio receptor.

The serotype (Inaba or Ogawa) was apparently irrelevant as to which hemagglutinin was expressed. The fact that L-fucose gave complete HAI of classical strains and D-mannose gave HAI of El Tor strains argues against the normal presence of both hemagglutinins on the same strain. Instead, these findings suggest either a genetic or regulatory difference in hemagglutinin expression, and studies are in progress to distinguish between these possibilities by testing mutants of classical and El Tor bacteria lacking the normal biotype-associated hemagglutinin. One exceptional classical *V. cholerae* strain, strain 569B, did exhibit both fucose-sensitive HA and mannose-sensitive HA. This suggests that this

strain atypically expresses both hemagglutinins.

The results described in this paper support our previous finding (9) that immunoglobulin plays only a minor role in the inhibitory activity of "normal" Swedish human milk observed for both classical *V. cholerae* adhesion and El Tor *V. cholerae* adhesion to erythrocytes; obviously, antibodies may play a much greater role in milk from women in areas where cholera is endemic (9). Instead, the main activity was found to be heat stable and also to bind to concanavalin A, properties consistent with a glycomolecule. Molecular weight fractionation separated the HAI activity against classical *V. cholerae* into two approximately equal fractions, one with a high molecular weight (>10,000) and the other with a molecular weight of 1,000 to 10,000; the heat-stable activities of both fractions were destroyed by periodate oxidation, supporting their glycocompound nature. On the other hand, about 80% of the HAI activity for El Tor vibrios was found in the high-molecular-weight fraction (fraction A) and 15 to 20% was found in the <1,000-dalton fraction (fraction C).

Otnaess and Ørstavik (15) recently found that they could extract *E. coli* enterotoxin inhibitory activity from milk with a chloroform-methanol mixture, indicating that this activity was of a glycolipid nature (GM1 ganglioside?). We did not find evidence for any glycolipid-associated inhibitory activity for *V. cholerae* erythrocyte adhesion. Lipid extractions did not remove the HAI activity, and both acid and neutral glycolipid preparations of milk lacked HAI activity for either of the two *V. cholerae* biotypes. Instead, we found that the high-molecular-weight HAI activities for both classical and El Tor vibrios resided on glycoproteins. Thus, saturated ammonium sulfate completely precipitated all of the high-molecular-weight HAI activity, and pronase digestion of fractin A transferred all of the high-molecular-weight HAI activity to a smaller (<10,000-dalton) fraction, which is consistent with degradation to a glycopeptide(s) with retained HAI activity.

Human milk is a rich source of oligosaccharides. Many of these oligosaccharides are fucosyl or fucosyl sialyl derivatives of either lactose or lacto-*N*-tetraose (12). Hence, we assume that one or more of these fucose-containing oligosaccharides are responsible for the HAI activity of fraction B observed for classical *V. cholerae*. The low molecular weights of the components of fraction B make it less likely that they are either glycoproteins or glycolipids (which would form larger micelles); furthermore, the inability of ammonium sulfate to precipitate any HAI activity of this fraction does not support a glycoprotein-mediated effect, and, as mentioned above, no HAI activity was found in glycolipid fractions of milk. HAI activity for El Tor *V. cholerae* was almost absent in fraction B, which is consistent with the fact that to date no mannose-containing oligosaccharides have been found in human milk (12). Finally, the HAI activity of fraction C (<1,000 daltons) for El Tor vibrios might represent free mannose.

Thus, our results support the hypothesis (9) that the main inhibitory activity for bacterial cell adhesion in nonimmune milk, as studied with HA by *V. cholerae*, is due to glycocompounds, which probably are structurally identical or closely related to the cell-bound receptors for the respective bacterial adhesins. These findings may have potential biomedical importance in several ways.

First, they suggest the possibility that isolation and characterization of the inhibitory carbohydrates from milk, and possibly from other secretions as well, might be a useful approach to the identification of the natural cell-bound receptors for classical and El Tor *V. cholerae* strains. Alternatively, the receptor structure might be deduced from determinations of the adhesion inhibitory activity of isolated milk oligosaccharides of known structures; this approach appears to be especially attractive for the identification of the receptor structure for classical *V. cholerae* in view of the many known fucosyl oligosaccharides from milk that are available in pure form. This would be analogous to the fact that most of our structural knowledge about blood group antigens was first obtained from studies of soluble antigens in secretions rather than from studies of extracted cell-bound antigens.

Second, our findings may have relevance in relation to the host defense of newborn infants. Enteric infections are especially frequent at the time of weaning and in infants not receiving human milk (14). Several studies have indicated that breast milk protects against enteric infections (4, 6). Daily administration of human colostrum or milk to non-breast-fed, hospitalized infants has been reported to reduce markedly the incidence and severity of diarrheal disease among infants (13). This protective effect has been ascribed mainly to the high content of immunoglobulin, especially secretory immunoglobulin A, in these secretions. Indeed, studies in Bangladesh have suggested a protective role for children of specific breast milk antibodies to cholera antigens (R. Glass et al., submitted for publication). However, our results suggest that the receptor-like glycocompounds identified by us also should be considered as candidate factors in milk that might contribute to protecting newborn children against *V. cholerae* and other enteric infections.

Third, if inhibitory receptor-like glycocom-

pounds like those now identified in milk are also present in saliva and gastrointestinal juice, they could form the basis of a system contributing to defense against mucosal infections. Studies are in progress to test these various hypotheses.

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