Inhibition of Adhesion of S-Fimbriated *Escherichia coli* to Epithelial Cells by Meconium and Feces of Breast-Fed and Formula-Fed Newborns: Mucins Are the Major Inhibitory Component

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Summary: We investigated the ability of meconium, feces from human milk-fed (HMF) newborns, and feces from formula-fed (FF) newborns to inhibit adhesion of S-fimbriated *E. coli* to human buccal epithelial cells. S-fimbriae are a common property of *E. coli* strains causing sepsis and meningitis in neonates. Meconium had the highest content of neuraminic acid and the strongest inhibitory effect on bacterial adhesion. HMF also exerted high inhibitory activity while FF was markedly less active: To achieve inhibitory effects comparable to HMF a sixfold amount of FF was required. Glycoproteins from excretions were separated by gel chromatography. Fractions obtained were analyzed for adhesion-inhibiting activity. In all excretions analyzed, the mucin-containing fraction could be identified as the major inhibitory component. Inhibition was probably mediated by specific interaction of this fraction with S-fimbriae, as shown by binding of isolated fimbriae on Western blots after electrophoretic separation of glycoproteins. In conclusion, our data support the view that the mucin-containing fraction from meconium and human milk exerts antibacterial functions by preventing adhesin-mediated binding of pathogenic bacteria to mucosal epithelia. **Key Words:** S-fimbriated *E. coli*—Inhibition of adhesion—Meconium—Feces of human milk-fed newborns—Feces of formula-fed newborns—Mucins.

Bacterial adhesion to mucosal epithelial surfaces is a prerequisite for manifestation of infections (1). Lectin-like adhesins on fimbriae mediate colonization of eukaryotic tissues by recognizing carbohydrate receptors on their surface (2).

Neonatal sepsis and meningitis are still major problems in pediatric infectious diseases. In a retrospective study from 1981, *E. coli* was responsible for 30–40% of cases in the United States (3,4). S-fimbriae and K1 capsule type are major components of *E. coli* strains causing sepsis and meningitis during the neonatal period (5). These adhesins bind to sialyl-α-2,3-galactoside structures (6).

In the newborn, the gastrointestinal tract and the oropharynx seem to be the reservoir for *E. coli* that can invade the bloodstream and, subsequently, the central nervous system (7–9). Meconium and feces of human milk-fed (HMF) infants have a high content of neuraminic acid-containing structures. We compared the ability of meconium and feces of human milk-fed and formula-fed (FF) newborns and different fractions of these materials to inhibit adhesion of S-fimbriae carrying *E. coli* to human buccal epithelial cells.

MATERIALS AND METHODS

Bacteria

Adhesion experiments were carried out with *E. coli* strain HB101 carrying the recombinant plasmid

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(pANN 801-4) that encodes S-fimbriae (10). In a few preliminary experiments, strain IH 3084 (kindly provided by Dr. Korhonen, University of Helsinki, Finland), isolated from a newborn with meningitis and carrying S-fimbriae, was used. Since expression of S-fimbriae in this strain was unstable in vitro, this strain did not appear to be useful for further experiments.

Bacteria were grown on NB agar supplemented with 0.1% glucose and tetracycline (30 μg/ml). After an 18 h incubation period at 37°C, bacteria were harvested with ice-cold 20 mM sodium borate buffer at pH 9.0 and washed twice with the same buffer. Following adjustment to 10^{10} bacteria/ml, 1 ml of the suspension was labeled with 100 μg of fluorescein isothiocyanate (FITC, Sigma Chemicals Co., St. Louis, MO, U.S.A.) as described by Parkkinen et al. (11). The expression of S-fimbriae in the strains was confirmed by inhibition assays as previously described (12).

Isolation of Buccal Epithelial Cells

Buccal epithelial cells (BECs) were obtained from healthy adult nonsmokers by scraping mucosa with a spoon-shaped spatula several times. Cells were washed off the spatula with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.5% methyl-α-D-mannoside, washed three times, and microscopically adjusted to 1×10^5 cells/ml.

Binding of Bacteria to Buccal Epithelial Cells and Preincubation of Bacteria with Inhibitors

Five hundred microliters of bacterial suspension or bacteria/inhibitor mixtures adjusted to 1×10^8 cells/ml was added to an equal volume of BEC suspension (1×10^5/ml) and incubated for 60 min in an ice bath under gentle shaking. Epithelial cells were washed three times with PBS (centrifugation for 5 min at 4°C and $120 \times g$) before microscopic evaluation. Fifty epithelial cells were analyzed for each experiment. All experiments were done in duplicate.

For inhibition studies, bacteria were preincubated with potential inhibitors for 15 min in an ice bath under gentle shaking. Inhibitor concentrations/ volumes stated below refer to the 15 min preincubation period.

The following inhibitors and volumes were used: meconium samples were collected from five newborns (first excretion, first day). One gram (wet weight) of each sample was homogenized and diluted in 15 ml of 0.9% NaCl. After centrifugation ($5,000 \times g$) to remove solid components, the supernatant was filtered through a paper filter and diluted with six parts of NaCl. Forty, 80, 160, and 320 μl/ml of inhibitor mixtures were used. The same procedure was applied to feces from five human milk-fed and five formula-fed (adapted, Pre Aptamil, Milupa, Germany) newborns (6 days of age).

For other inhibition experiments, 200, 400, 800, and 1,000 μg of the glycoprotein fractions from meconium, HMF, and FF per 1 ml of inhibitor mixture were tested. These fractions were obtained after phenol–saline extraction of excretions with subsequent dialysis against distilled water (48 h, 4°C) and lyophilization (13).

The content of N-acetylneuraminic acid (NeuAc) and protein of each sample (raw suspension and sialoglycoprotein fraction) for the inhibition assay were determined according to Warren (14) and Lowry, respectively (15). Furthermore, 38 meconium, 17 HMF, and 18 FF samples were analyzed. As controls, asialoglycoproteins were prepared from the glycoprotein fractions by acid hydrolysis (0.1 M HCl at 80°C for 1 h) and used in the binding assay.

Gel Chromatography of Sialoglycoproteins

Lyophilized sialoglycoproteins were separated according to their size on a Sephacryl S-300 column (100×2.5 cm), eluted with 4 M guanidine hydrochloride, pH 7.0, containing 1 mM ethylenediamine-tetraacetic acid (EDTA) and 1 mM Na$_2$PO$_4$. Five milliliter fractions were collected. Carbohydrates were detected in aliquots by the phenolsulfuric acid procedure of Dubois et al. (16) at 485 nm. Additionally, the protein content was recorded by spectrometry at 280 nm.

Pooled peak fractions were dialyzed against H$_2$O, lyophylized, and then used at different concentrations as inhibitors in the binding assay. Aliquots of these fractions were further analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and staining with Coomassie blue (CB), periodic acid–Schiff reagent (PAS), and wheat germ agglutinin (WGA). The content of NeuAc (14) and protein (15) was determined.

Isolation and Purification of S-Fimbriae

S-fimbriae were isolated essentially according to the procedure described by Salit and Gotschlich

(17) with the modification of Wevers et al. (18). The purity (homogeneity) of the preparation was confirmed by SDS–PAGE and examination by electron microscopy.

Western Blot Analysis with Isolated Fimbriae

Glycoproteins were separated (by PAGE) after preincubation in 100 μl of sample buffer containing 0.3% SDS and 3% 2-mercaptoethanol for 10 min at 90°C. For WGA staining, 1 mg was used, and for binding of purified fimbriae, 3 mg was used. Electrophoresis of sialoglycoproteins from meconium, HMF, and FF was performed in polyacrylamide gels (3–13%) in the presence of SDS (0.1%). Gels were fixed and stained with CB or PAS reagent. Electroblotting onto nitrocellulose (0.45 μm) was performed in Tris (25 mM), glycine (192 mM), and methanol (20%).

For WGA staining, the nitrocellulose membranes were immersed in PBS at pH 7.2 containing 5% (w/v) BSA to block nonspecific binding sites and overlayed with a solution of WGA–peroxidase conjugate (5–10 μg/ml) diluted in 0.5% BSA/PBS. After incubation for 1 h at room temperature and three successive washing steps (0.5% BSA/PBS), the membranes were developed in PBS/methanol (17% v/v) containing 1-chloro-4-naphthol (0.055%) and hydrogen peroxide (0.008%). Optimum staining was obtained after 30 min in the dark.

For binding of purified fimbriae to transblots, the strips were blocked as mentioned above. Next, the membranes were overlayed with fimbriae suspended in PBS (125 μg of protein/ml) for 17 h at 4°C. For immunostaining, membranes were incubated for 1 h at 4°C with monoclonal antibody A 21356 A1 (1:1,000) (kindly provided by Dr. K. Jann), which recognizes S-fimbriae (19). After washing, alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:1,000) (Dako, Hamburg, Germany) was added for 30 min at room temperature and washed four times with PBS. Membranes were developed with 5-bromo-4-chloro-3-indolylphosphate sodium salt and Nitro blue tetrazolium chloride (Serva, Heidelberg, Germany) for 15 min at 37°C.

RESULTS

Adhesion of S-Fimbriated *E. coli* to Human Buccal Cells

Fluorescence microscopy of buccal epithelial cells after incubation with FITC-labeled S-fimbri-

ated *E. coli* strain HB 101 (pANN 801-4) showed that 15–30 bacteria had bound to one epithelial cell if no inhibitor was used (12). Binding characteristics of the clinical isolate IH 3084 to buccal epithelia did not differ from that of the cloned strain HB 101 (pANN 801-4) (data not shown).

Content of *N*-Acetylneuraminic Acid and Protein in Meconium, HMF, and FF

The NeuAc and protein content of meconium, HMF, and FF are shown in Table 1. In all raw excretions, 20–25% of NeuAc was free neuraminic acid, and the rest was glycoconjugate bound.

Inhibition of Bacterial Adhesion to Human Buccal Epithelial Cells by Meconium, HMF, and FF

Figure 1A shows that the strongest inhibitory effect on adhesion of cloned S-fimbriated *E. coli* to human buccal epithelial cells was exerted by meconium, and the weakest by FF. In order to achieve 40% inhibition, 40 μl of meconium preparation was required. Comparable inhibitory effects of HMF required twice the volume, and FF a 12-fold greater volume.

Figure 1B shows a similar relationship between the NeuAc content and the extent of adhesion inhibition for both meconium and HMF. For FF, in contrast, a two- to threefold higher NeuAc concentration is required to achieve comparable inhibitory effects.

Using isolated glycoprotein fractions from meconium, HMF and FF inhibition in relation to the NeuAc concentration resembled the FF raw preparation (data not shown).

TABLE 1. *Content of* N*-acetylneuraminic acid and protein in meconium, HMF, and FF[a]*

	NeuAc (mg/g of wet weight)[b]	Protein (mg/g of wet weight)[c]
Raw meconium (n = 43)	15.2 ± 2.6	3.5 ± 5.4
Raw human milk feces (n = 22)	7.9 ± 2.6	1.2 ± 5.4
Raw formula feces (n = 23)	3.4 ± 1.4	1.9 ± 6.9

[a] Means ± SD are given.
[b] NeuAc was measured according to Warren (14).
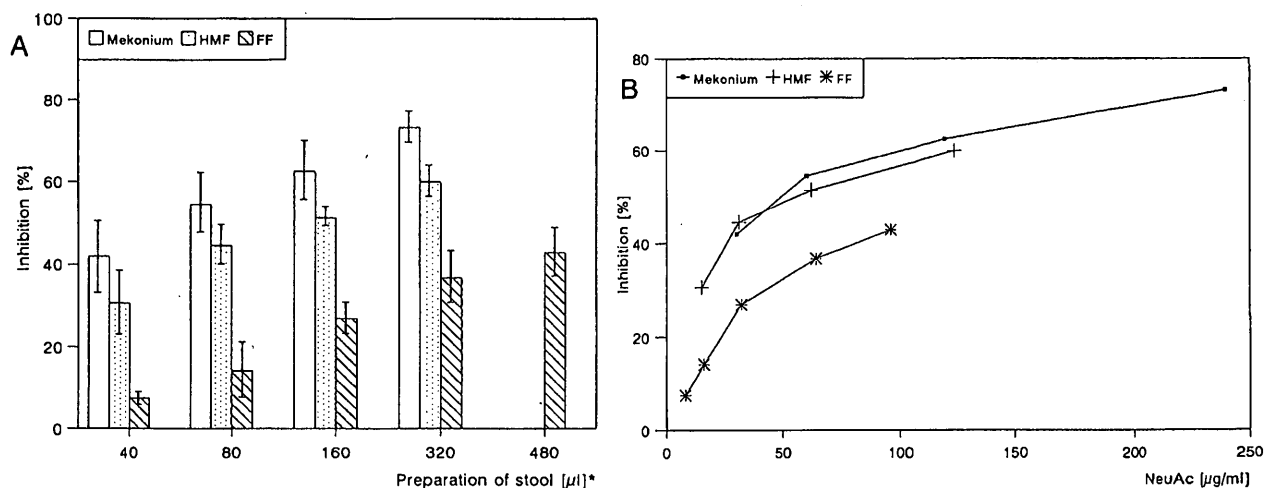[c] Protein was measured by the Lowry method (15) using bovine serum albumin (BSA) as standard.

FIG. 1. Inhibition of bacterial adhesion [*E. coli* HB 101 (pANN 801-4)] to human buccal epithelial cells by raw preparations of meconium and feces of breast-fed and formula-fed newborns. **A:** Inhibition of bacterial adhesion in relation to the volume of raw preparations. **B:** Inhibition of bacterial adhesion in relation to the NeuAc concentration. For preparation of the suspension, refer to the Materials and Methods section. Means and standard deviations are given, *n* = 5. Four hundred eighty microliters was used additionally, because with lower volumes less than 40% inhibition was achieved (*n* = 3). The NeuAc content of each stool preparation was calculated from five measurements: meconium, 746 ± 33 μg/ml; HMF, 385 ± 114 μg/ml; FF, 200 ± 62 μg/ml. Volumes of stool preparations refer to 1 ml of inhibition mixture.

Desialination reduced the ability of glycoproteins (1 mg/ml) to inhibit adhesion to 10–15%.

Gel Chromatography of Glycoproteins

The major peaks (A–C) obtained after gel chromatography of feces glycoproteins were pooled separately, as shown in Fig. 2A–C. Pooled fractions were dialyzed against distilled water and lyophilized. A and C were tested for inhibitory activity in the bacteria binding assay. Concentrations of inhibitors leading to approximately 50% inhibition of bacterial adhesion are shown in Table 2.

High-molecular-weight fraction A (void volume >200 kDa) showed the strongest inhibitory activity. Using pooled fractions of A, 50% inhibition could be achieved with 250 μg/ml (meconium) or 500 μg/ml (HMF and FF). For pooled fractions of C, 750 μg/ml of inhibition mixture was required. The absolute NeuAc content of fraction A was in the range of 5–16% of fraction C. Sialinization, however, of glycoproteins in fraction A with a NeuAc/protein ratio of 20:1–≥60:1 was markedly higher compared to fraction C with a ratio of 1:1–1.3:1.

Pooled fractions were further analyzed by SDS–PAGE and Western blot (Fig. 3). Staining of the gels with CB and PAS of pools A revealed high-molecular-mass glycoproteins (>200 kDa) positive for PAS but negative for CB. This band was WGA

positive on Western blot analysis and was thus classified as mucin because of molecular mass and staining behavior. Fraction C was also negative for CB staining and showed weak PAS- and WGA-positive bands corresponding to sialoglycoproteins of lower molecular weight. Blots of meconium fractions are shown. Similar staining behavior was seen with HMF and FF fractions. The staining pattern of pool B was comparable to pool C (data not shown).

Binding of Isolated S-Fimbriae to Transblots

In order to characterize better the major component responsible for adhesion inhibition, isolated fimbriae were incubated with nitrocellulose strips containing separated glycoproteins (Fig. 4). Fimbriae predominantly bound to the WGA- and PAS-positive mucin-containing bands.

DISCUSSION

We have recently shown that S-fimbriated *E. coli* plays a major role in neonatal sepsis and meningitis (5) by adhering to human buccal epithelial cells (12). In the present study, we investigated the ability of meconium, HMF, and FF to inhibit adhesion of S-fimbriated *E. coli* to epithelial cells.

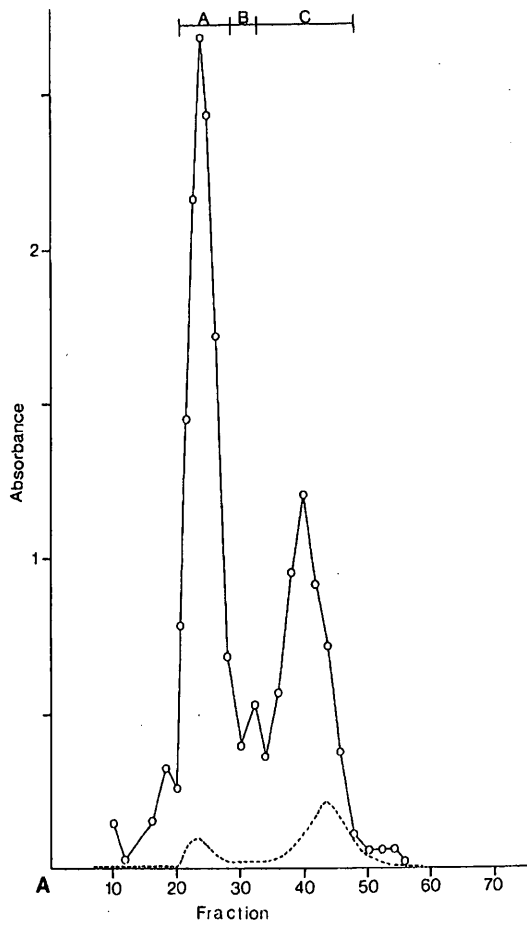The most relevant system to study such antiad-

FIG. 2. Gel chromatography profile (Sephacryl S-300) of glycoproteins from meconium, HMF, and FF. Absorbance was measured at 280 nm () and 485 nm (O-O). Eluted fractions were pooled as indicated: **A:** 50 mg pooled from first five excretions was applied; **B:** 40 mg of pooled stools from five breast-fed 6-day-old newborns was applied; **C:** 60 mg of pooled stools from five formula-fed 6-day-old newborns was applied.
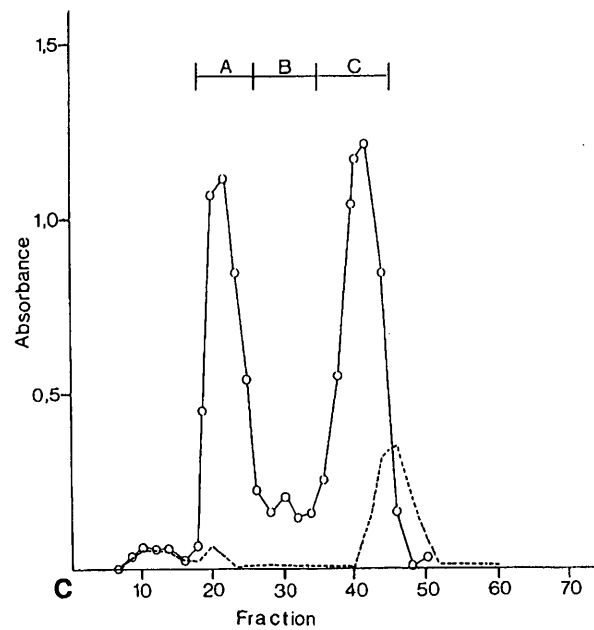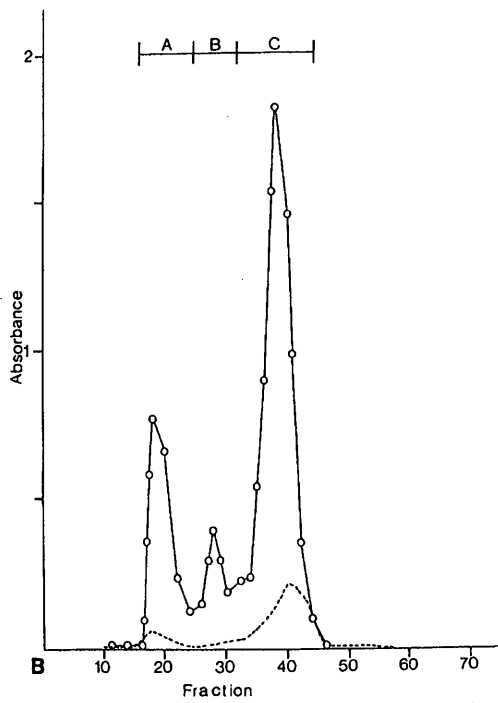
TABLE 2. *Fifty percent inhibition of bacterial adhesion to human buccal epithelial cells by pooled glycoprotein fractions (A–C) of meconium, HMF, and FF after gel chromatography on Sephacryl S-300*[a]

Inhibitor (μg/ml)	NeuAc concentration of inhibitor present in inhibition mixture (μg/ml)	Protein concentration of inhibitor present in inhibition mixture (μg/ml)
Meconium		
Pool A, 250	13.9	[b]
Pool B,[c] nd		
Pool C, 750	78.8	60.6
Human milk feces		
Pool A, 500	9.3	0.5
Pool B, 750	18.3	16.1
Pool C, 750	63.5	67.0
Formula feces		
Pool A, 500	15.2	0.5
Pool B, 750	41.8	15.3
Pool C, 750	102.0	83.8

[a] Means of duplicate determinations are given.

[b] Below the detection limit of the Lowry method (15), i.e., <1 μg/ml.

[c] Pool B could only be analyzed for the content of N-acetyl-neuraminic acid (56.9 μg/ml) and protein (10.0 μg/ml) because little material was obtained.

hesive properties of excretions would, of course, be viable intestinal epithelia from newborns. Unfortunately, these are difficult to obtain. Human intestinal cell lines as a possible alternative undergo morphological and functional changes after a few passages in tissue culture (20).

Adhesive properties of a given bacterial strain vary depending on tissues used (21). We used buccal epithelial cells for two reasons: first, oral epithelia are an easily accessible part of the gastrointestinal tract and, second, the oropharynx and the intestine both serve as a natural entry route for invasive pathogenic *E. coli* (7,9). Adhesion of S-fimbriated *E. coli* to buccal epithelial cells is, similarly to type I fimbriae-carrying *E. coli*, age independent (22,23). Therefore, epithelial cells from adults were used as a model to study bacterial adhesion.

Meconium in our study showed the highest inhibitory activity against adhesion of S-fimbriated *E. coli* to buccal epithelial cells. Its high content of neuraminic acid-containing glycoproteins with O-linked carbohydrate chains of the mucin type is well known (24–27).

The mucin-containing fraction from meconium, HMF, and FF are the major components in glycoprotein fractions separated by gel chromatography (pool A) for both inhibition of bacterial adhesion and binding of isolated fimbriae to transblots.

Smaller sialoglycoproteins (pool C < 60,000 MG), in this respect, play only a minor role. Meconium mucins originate from fetal intestine and swallowed amniotic fluid mucins (28), HMF mucins from intestine and milk (29,30), and FF mucins from intestine only (31).

The anti-infectious potential of mucins in the prevention of bacterial adhesion to epithelial tissues has been documented in recent studies (32,33,34). This particular function of mucins is related to their unique structural properties, especially to their conjugated and often highly sialylated carbohydrates, which express a multitude of receptor-analogous
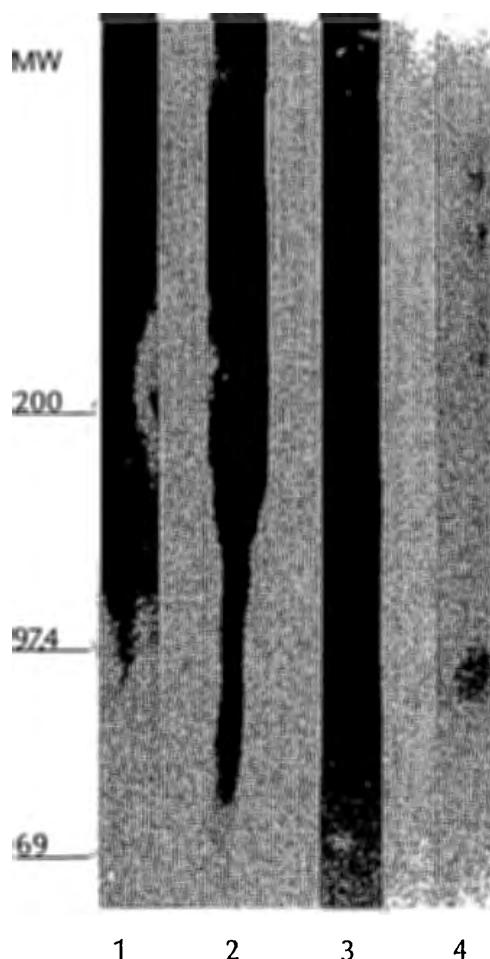

FIG. 3. Polyacrylamide gel electrophoresis and Western blot analysis of pooled fractions A and C of HMF glycoproteins separated by gel chromatography (see Fig. 2B). Fraction A: lanes 1 and 2; fraction C: lanes 3 and 4. Freeze-dried samples containing 30 μg of protein (Lowry) per lane were applied. Lanes 1 and 3 are gels stained with PAS; lanes 2 and 4 are Western blots, stained with WGA. Rainbow protein molecular weight markers (Amersham, Braunschweig, Germany) were used as standards.
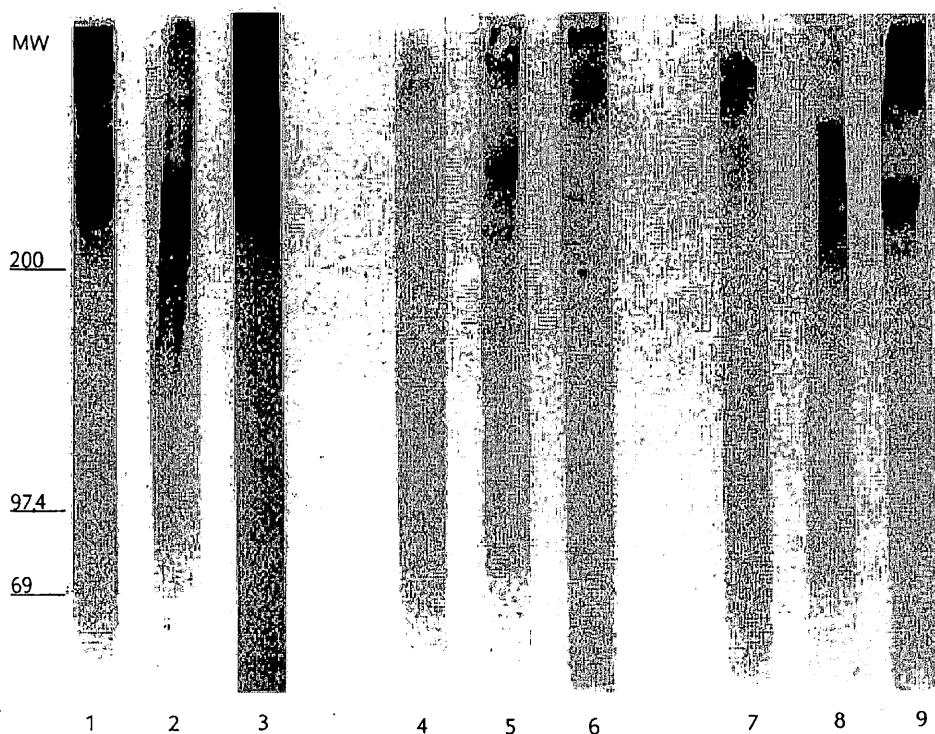
FIG. 4. Binding of WGA and S-fimbriae to glycoproteins after separation by SDS–PAGE and blotting to nitrocellulose. Lanes 1–3 contain glycoproteins from meconium, lanes 4–6 contain glycoproteins from stools of breast-fed newborns, and lanes 7–9 contain glycoproteins from stools of formula-fed newborns. Lanes 1, 4, and 7 are gels stained with PAS; lanes 2, 5, and 8 are Western blots stained with WGA; and lanes 3, 6, and 9 are immunostained for S-fimbriae after incubation with nitrocellulose strips as described in the Materials and Methods section. Rainbow protein molecular weight markers (Amersham, Braunschweig, Germany) were used as standard.

structures for bacterial adhesins. Recently, it has been reported that in receptor-binding assays, milk fat globule mucins have inhibitory activity against human immunodeficiency virus (35).

Neeser et al. (36,37) have been able to demonstrate a significant inhibitory effect of meconium glycopeptides on colonization factor antigen (CFA) I and II-mediated hemagglutination by *E. coli* and CFA II-mediated adhesion to the enterocyte-like differentiated HT 29. Although adhesins of CFA I and II complexes also recognize neuraminic acid-containing structures (38), meconium preparations used in the experiments of Neeser et al. (36,37) were able to inhibit hemagglutination or adhesion to HT 29 cells even after desialination. This finding is in contrast to our results obtained with S-fimbriated *E. coli:* desialination of glycoproteins from all sources markedly reduced the adhesion-inhibiting capacity by 85–90%.

On biochemical analysis of the stool preparations, we noticed that raw preparations of meconium and HMF had similar inhibitory activities on

the basis of their NeuAc content. In contrast, raw preparations of FF with comparable NeuAc concentrations were two- to threefold less active in comparison to meconium and HMF. Using isolated glycoproteins from meconium, HMF, and FF, this difference was markedly less pronounced. Components other than the glycoprotein fractions may be responsible for this effect, e.g., neuraminic acid-containing glycolipids or oligosaccharides that were not recovered during the process of glycoprotein preparation (39–41). This would also explain differences between the NeuAc content of raw preparations and glycoproteins. However, in earlier studies [Schroten et al. (23)], we were able to show that the oligosaccharide fraction from human milk does not inhibit adhesion of S-fimbriated *E. coli* to buccal epithelial cells.

After separation of glycoproteins, the mucin-containing high-molecular-weight fraction A of all stool preparations was most effective with respect to inhibition of bacterial adhesion. This finding correlates well with the high degree of sialination of

glycoproteins in fraction A (approximately 20- to 60-fold compared to fraction C). Predominantly, neuraminic acid covalently linked to mucins correlated with the inhibitory effect, not the total (including nonmucin) neuraminic acid content. These mucins are constituents of the glycoprotein fraction.

If equal volumes of meconium and HMF were assayed for adhesion-inhibiting capacity, meconium was clearly more effective. This could, at least in part, be due to the higher content of mucin-bound neuraminic acid. FF exhibited little inhibitory effects compared to meconium and HMF. This cannot be solely explained by the known low content of neuraminic acid in raw material (42). Structural differences of mucins in different excretions could play a role in addition to their relative content in the glycoprotein fraction.

The results presented in this study support the view that mucins present in breast milk contribute to antibacterial mechanisms present along the entire intestine.

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