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Glycoprotein Glycans That Inhibit Adhesion of *Escherichia coli* Mediated by K99 Fimbriae: Treatment of Experimental Colibacillosis

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Calf diarrhea due to infection by enterotoxigenic *Escherichia coli* was treated by administration of glycoprotein glycans derived from bovine plasma. The glycan moieties of the nonimmunoglobulin fraction of plasma mimicked the oligosaccharide moiety of intestinal receptors recognized by K99 pili. These glycoprotein glycans inhibited adhesion of *E. coli* K99$^+$ ST$^+$ to erythrocyte glycoconjugates in vitro, and they protected colostrum-deprived newborn calves against lethal doses of enterotoxigenic *E. coli* (10^{10} bacteria). Adhesion of bacteria to the intestines (duodenum, jejunum, and ileum) was significantly reduced (by 2 orders of magnitude) in treated calves.

The pathogenicity of enterotoxigenic *Escherichia coli* (ETEC) in infectious diarrhea in calves is due, in large part, to the action of enterotoxins (37). However, it is now known that adhesion of bacteria to the host also plays an important role in bacterial virulence (34). Adhesion of bacteria is an early process in the development of an infection which involves binding of bacterial lectins, such as K99, F41, and FY pili (10, 11, 18), to intestinal glycoconjugate receptors. Various sialoconjugates have been proposed as receptor sites for K99 pili. The carbohydrate moiety of the sheep erythrocyte receptor appears to resemble ganglioside GM2: GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer (17). K99 pili also recognize the oligosaccharide NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc of human erythrocyte glycophorin A (4, 23), and NeuGc(α2-3)Galp(β1-4)Glc(1-1) has been identified in horse erythrocytes (32, 36). These structures have the minimal characteristics of receptors used by pathogenic agents to adhere to intestinal epithelium.

Treatment of bacterial infections by competitive inhibition of bacterium-receptor interactions (20, 21, 29, 41) or toxin-receptor interactions (20, 33, 40) has been proposed. We have demonstrated in vitro that various bovine plasma glycoproteins inhibit adhesion of ETEC to sheep erythrocytes (27) and mucosal glycoproteins (28). The present study was designed to confirm these in vitro results in vivo. The effect of inhibition of bacterial adhesion by plasma glycoprotein glycans was evaluated with experimental ETEC K99$^+$ colibacillosis in calves. Oral treatment with analogs of mucosal and enterocyte receptors may thus represent a new approach to the therapy of bacterial infections.

(Preliminary accounts of this work have appeared previously [M. Mouricout and R. Julien, Abstr. XXIII World Veterinary Congress, Montreal, Canada, abstr. no. 7.15.1, 1987].)

MATERIALS AND METHODS

***E. coli* strains.** *E. coli* B41(O101: H, K99, F41) (Institut Pasteur, Paris, France) was used for in vitro tests. All tests were performed with 18-h cultures of *E. coli* grown on solid Minca medium Polyvitex (Bio-Mérieux, Charbonnières-les-Bains, France) at 37°C.

The K99$^+$ ST$^+$ Nal^r *E. coli* strain used to induce experimental colibacillosis (kindly provided by J. L. Martel, Laboratoire National de Pathologie Bovine, Lyon, France) was isolated from diarrheic calves. This strain is resistant to nalidixic acid (26), and this property was used as a specific marker to detect and count the pathogenic bacteria in the overall intestinal flora. The strain was grown on Minca medium Polyvitex containing nalidixic acid (40 μg/ml; Boehringer, Mannheim, Federal Republic of Germany) for 18 h at 37°C.

The presence of K99 pili was tested by an agglutination test using specific antisera (Iffa-Mérieux, Lyon, France).

Glycoprotein glycans from total plasma. The method of preparation of adhesion inhibitors is patented. Briefly, plasma glycoproteins were hydrolyzed by 1% (wt/wt) purified *Bacillus licheniformis* subtilisin A for 6 h at 50°C and pH 8 (2, 3). The fractions containing inhibitor components were separated from enzyme and low-molecular-weight fractions (peptides and free sugars) and concentrated by membrane ultrafiltration (molecular cutoffs, 10^3 and 10^4). Ten grams of two dried glycopeptide preparations (p1 and p2) contained peptides (p1, 6.55 g; p2, 5.80 g; Kjeltec Auto 1030 Analyser; Tecator, Höganäs, Sweden); oligosaccharides containing sialic acids (p1, 220 mg; p2, 270 mg); and sodium, potassium, and calcium salts (p1, 1.24 g; p2, 1.52 g; atomic absorption spectrometer Spectra A10; Varian, Mulgrave, Australia). The standard dose was defined in terms of the carbohydrate content determined by the method of Dubois et al. (14).

Glycoprotein glycans from fractionated plasma. Bovine plasma was defibrinated and desalted by gel filtration on a Trisacryl GF05 column (18 by 40 cm) (Réactifs IBF, Villeneuve-la-Garenne, France) with a flow rate of 6.5 liters/h with 50 mM Tris hydrochloride, pH 8.8.

Protein material was fractionated by automated stepwise ion-exchange chromatography on a DEAE-Trisacryl M column (18 by 40 cm; Réactifs IBF) with a flow rate of 11 liters/h using 50 mM Tris hydrochloride buffer (pH 8.8) for step 1, 50

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mM Tris hydrochloride–40 mM NaCl for step 2, and 50 mM Tris hydrochloride (pH 8)–100 mM NaCl for step 3. Isolated fractions were hydrolyzed with 1% *Bacillus subtilis* protease (subtilisin A; EC 3.4.21.14; Sigma Chemical Co., St. Louis, Mo.) for 0.5 to 6 h at 50°C. The degree of hydrolysis was determined as described by Alder-Nissen (1): % hydrolysis = (no. of enzymatically cleaved peptide bonds/total no. of chemically cleaved peptide bonds) × 100.

The presence of anti-*E. coli* K99 antibodies was tested by specific agglutination of several *E. coli* strains (27) (one optical density unit, 200 μl) by 200 μl of twofold-diluted cattle serum (from 50 mg/ml) or fractionated plasma (10 mg/ml).

MRHA and its inhibition. A 200-μl volume of a B41 bacterial suspension (10^8/ml) induced agglutination of sheep erythrocytes (10^8 cells per ml) in 200 μl of 0.15 M phosphate-buffered saline (pH 6.8) containing 0.1 M α-methylmannoside (PBSM). After 1 h at 4°C, the nonagglutinated erythrocytes (RBC_f) in 10-μl supernatant samples obtained during shaking (450 rpm) were counted in 20 ml of Isoton (Coultronics, Margency, France) with an electronic particle counter (tube orifice, 100 μm; Coulter Counter ZM; Coultronics). Total hemagglutination was determined from the residual value of RBC_f in the presence of bacteria, and 0% mannose-resistant hemagglutination (MRHA) was determined by the number of RBC_f in PBSM.

Inhibition of hemagglutination was obtained by incubation of bacteria with glycoprotein glycan preparations (200 μl, from 0.1 to 10 mg/ml in amino acid equivalents) for 5 min at 4°C in PBSM. Erythrocytes were then added, and the adherence test was performed as described above. Inhibition of adhesion (%) was expressed by the following relationship: [(RBC_f in inhibition test − RBC_f in 100% MRHA)/(RBC_f in 0% MRHA − RBC_f in 100% MHRA)] × 100.

Calf inoculation procedures. Friesian calves were colostrum deprived to eliminate the effects of maternal antibodies (19). Calves were collected from farms at birth. On their arrival in the laboratory, immunoglobulin G (IgG) status was checked by electrophoretic examination of serum and by measurement of immunoglobulin levels with the Agglutinade calf immunity test (Ab-Ag Laboratories, Ely, United Kingdom). The size of the *E. coli* $K99^+$ ST^+ Nal^r inoculum prepared in 0.9% NaCl solution (approximately 50 ml) was evaluated on the basis of its optical density (570 or 642 nm) and by dilution and plating of samples on appropriate media. The inoculum was then diluted to the desired concentration (10^7 to 10^{10} CFU). The inoculum was administered into the back of the mouth with a syringe when the calves were 2 to 8 h old.

Treatment of calves with glycoprotein glycans (or antiadhesins). Glycoprotein glycan preparations (p1 and p2; 250 mg of oligosaccharides) in water (approximately 500 ml) were administered orally as soon as the first signs of diarrhea appeared. Treatment was usually repeated for 3 days.

Control calves. Control calves were given water either without antiadhesins (calves 2, 3, 6, 10, 15, 17, and 23) or with commercial oral rehydrating agents (calves 24 and 25). The latter calves received oral rehydration at the dosage recommended by the manufacturer. In some experiments, calves 20 to 22 received plasma hydrolysates in which the oligosaccharide structures had been destroyed (27). These preparations had the same protein content as antiadhesin preparations p1 and p2.

Clinical findings and analyses. Calves were kept in clean and disinfected rooms, examined on challenge, and inspected at frequent intervals. Fecal pH, consistency of feces, and degree of dehydration were noted at each examination of all calves. Potassium in plasma and fecal potassium and sodium contents were determined in 11 calves (control calves 2, 3, 6, and 15 and treated calves 1, 4, 5, 7, 8, 9, and 12) by atomic absorption spectrometry.

Control calves were constantly monitored when death appeared imminent, and treated calves were slaughtered. Immediately after death, segments of duodenum, midjejunum, and ileum were collected for microbiological analyses. Adherent *E. coli* $K99^+$ ST^+ Nal^r populations were harvested as described by Smith and Huggins (38), by scraping the intestinal walls after the contents had been eliminated from each section. Numbers of *E. coli* organisms per gram of scrapings were determined by conventional plating techniques on Minca Polyvitex complemented with 40 μg of nalidixic acid per ml. The presence of rotavirus was tested with an enzyme-linked immunosorbent assay (Dakopatts K220; Flow Laboratories, Puteaux, France) (15).

Villosities (100 μl; 60 ± 6 μg [dry weight]) prepared as described by Girardeau et al. (18) were incubated with a bacterial suspension containing 10^8 bacteria per ml (200 μl) and 200 μl of phosphate-buffered saline (pH 6.8) for 15 min with slow shaking. After two washings with Krebs buffer, adherence was observed directly by microscopic examination under phase contrast (Leitz). Glycoprotein glycan preparations (0.25 to 5 mg/ml in phosphate-buffered saline) were then added to the adhesion system, and inhibition was assessed after 15 min of incubation.

Excretion of the challenge strain. Calves 1 and 2, which received 2 liters of colostrum each, and colostrum-deprived calf 3 were infected within 6 h of birth with 10^{10} *E. coli* organisms. They were treated with antiadhesins as described above (500 mg/day). One 12-h-old calf (calf 4) given colostrum to check survival without treatment (19, 25) was challenged with the same dose of *E. coli*. Feces were collected from both groups during 50 days, and pathogenic bacterial populations were measured. All calves were fed milk during the observation period.

RESULTS

Evaluation of the inhibitory capacity of glycoprotein glycans. The inhibitory capacities of the different molecular species of bovine blood antiadhesin preparations were determined by hemagglutination assays with a reference strain (*E. coli* B41).

Defibrinated plasma was separated into three fractions by ion-exchange chromatography. G2 immunoglobulins were recovered in fraction I (99.5%); fraction II was composed of β2 and IgG1 (52%), β1 (36.5%), and α globulins (10%); and fraction III contained albumin (71.5%), α globulins (20%), and β2 and γ globulins (8.5%). The presence of anti-*E. coli* antibodies and anti-K99 antibodies in bovine plasma was checked by using several strains of *E. coli* bearing K99 pili (27). Agglutination was observed for serum dilutions of 1/32 and 1/64, depending on the strain, and at dilutions of 1/320 for fraction I and 1/160 for fraction II.

Inhibition of MRHA (80% and 60% for fractions I and II, respectively) due to the presence of anti-K99 immunoglobulins or, more generally, anti-*E. coli* antibodies in fractions I and II (data not shown) disappeared after proteolysis (Fig. 1). The oligosaccharide structures of these immunoglobulins (Fig. 2) did not inhibit hemagglutination. Similarly, neither fraction II glycopeptides nor the albumin-derived peptides inhibited adherence to erythrocyte receptors. Only fraction III glycoprotein glycans inhibited bacterial adherence.

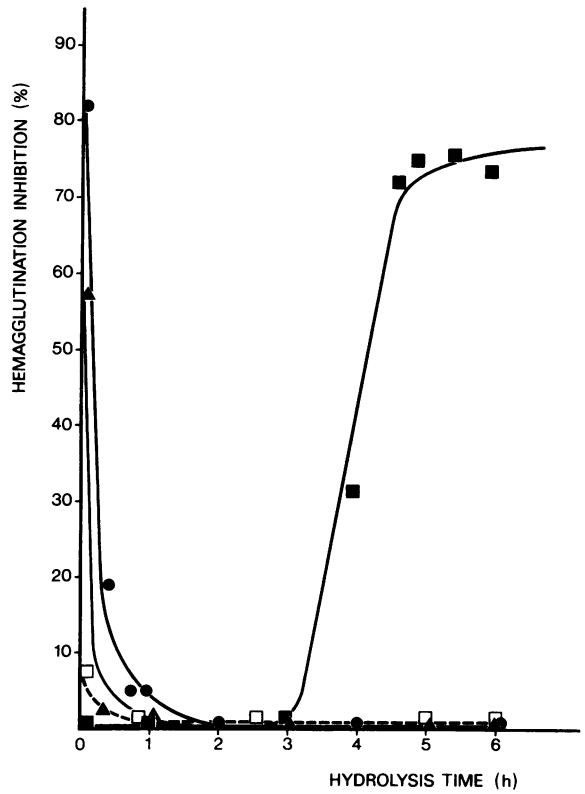

FIG. 1. Inhibitory capacities of native and hydrolyzed plasma glycoproteins. Three fractions, I (●; 5.2 μg of glycan per mg of peptide), II (▲; 13.6 μg of glycan per mg of peptide), and III (■; 13.6 μg of glycan per mg of peptide), separated on DEAE-Trisacryl were hydrolyzed by 1% subtilisin A at 50°C. The degree of hydrolysis was 35 ± 2% for the three fractions after 5 h of hydrolysis. Most of the glycopeptides had mean molecular weights below 10^4. The inhibitory capacities were measured by using a hemagglutination test (fraction I was at 0.25 mg/ml, and II and III were at 0.1 mg/ml of peptide). Bovine serum albumin (□) hydrolyzed under the same conditions was used as a control to measure the abilities of peptides to inhibit K99-mediated hemagglutination (at 10 mg/ml).

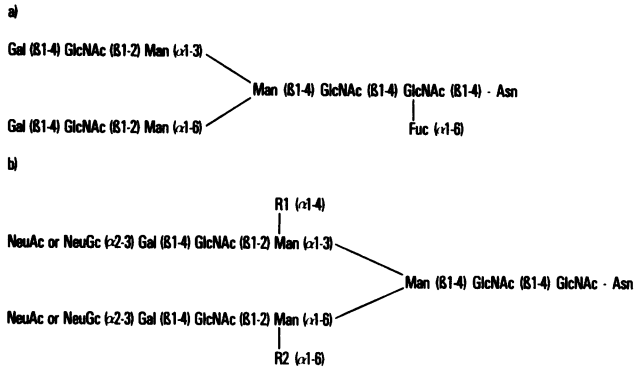

FIG. 2. Two examples of oligosaccharide structures obtained from glycoproteins in plasma. (a) Oligosaccharide isolated from bovine IgG (30). (b) Glycoprotein sialoglycan inhibitors of *E. coli* $K99^+$ adhesion. In biantennary structures, R1 = R2 = H; in triantennary structures, R1 = NeuAc or NeuGc(α2-3)Gal(β1-4)Glc NAc(β1-2) and R2 = H; in tetraantennary structures, R1 = R2 = NeuAc or NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-2).

Pathogenicity of the bacterial strain. Since a variety of bacterial properties, such as growth, chemotaxis, resistance to host defenses, and toxin production, play a role in virulence, we used a strain of *E. coli* that is representative of bovine pathology observed in the field. To determine the pathogenicity of this strain in experimental colibacillosis, calves were inoculated with bacteria (10^7 to 10^{10} organisms) within 8 h of birth. Bacterial colonization remained constant in the three sections of small intestine, and the infection was accompanied by fatal diarrhea (Table 1). For the rest of the experiments, the size of the inoculum was therefore fixed at 10^{10} to override individual variations. The animals were monitored for 72 h (Table 2), the time span generally used to evaluate antidiarrheal agents (R. H. Whitlock, J. E. Palmer, and T. J. Divers, Abstr. XXIII World Veterinary Congress, Montreal, Canada, abstr. no. 10-1-16, 1987).

Clinical course of infection after inoculation. Various functional disorders related to the diarrheal syndrome and the intensity of the infection were observed. In infected and untreated calves (controls), there was considerable loss of electrolytes due to the action of enterotoxins (Fig. 3). The pH of feces increased from a normal value of 5.5 to over 8 (Fig. 4), and hyperkalemia was consistently observed in severely dehydrated animals (Fig. 5). Diarrhea was less severe in calves treated with the glycoprotein glycan preparations (Table 2). Electrolyte loss was reduced (Fig. 3), and fecal pH returned to normal within 2 to 3 days (Fig. 4). Potassium levels in blood remained normal in these animals (Fig. 5). Treated calves survived the infection and were cured within 72 h in most cases (11 of 13 or 84.6%). One calf treated at a later time, 5 to 6 h after the onset of diarrhea, presented signs of dehydration and died.

Adhesion of ETEC to intestinal mucosa. A control group was not readily constituted, since most of the untreated calves died within 41 to 62 h postchallenge (Tables 1 and 2). Further experiments were performed with calves which received other types of treatment. Unfortunately, oral fluid therapy in two calves did not prevent death under the severe conditions of the experiment (colostrum-deprived calves inoculated with 10^{10} bacteria).

The beneficial effect of the treatment could, however, have been due to rehydration from the cocktail of carbohydrates and amino acids present in the antiadhesive material. To rule out this possibility, we compared the compositions of six commercial oral rehydratants and antiadhesin preparations. These preparations differed considerably in salt, sugar, and fluid contents. Only the quantities of amino acids (linked or free) were comparable (Fig. 6). We also administered total plasma hydrolysates without conservation of oligosaccharide structures (27) to calves 20 to 22; only 15 ± 2 and 25 ± 3% of MRHA was inhibited by these products

TABLE 1. Colonization of small intestines of control calves[a]

Inoculum size (no. of bacteria)	Onset of diarrhea (h postinfection)	Time of death after inoculation (h)	Log_{10} no. of bacteria/g in:		
			Duodenum	Jejunum	Ileum
10^7	27	44	6.62	9.41	9.17
10^8	22	72	6.28	8.71	8.78
10^9	18	42	7.29	9.51	9.04
10^{10}	12–18	41–52	6.27–8.04	7.84–9.97	7.47–9.94

[a] Inocula of 10^7 to 10^9 bacteria were given to one calf each, and an inoculum of 10^{10} was given to 12 calves. Pathogenic bacteria adhering to the intestinal mucosa were elevated in cultures of scrapings. Thus, only viable bacteria representative of infection (capable of multiplying and producing toxins) were counted.

TABLE 2. Clinical course of calves infected with *E. coli* K99$^+$ ST$^+$ Nal^r

Calf no. (sex)[a]	Age (h) at inoculation	Time (h) postchallenge of diarrhea		Time(s) (h) postinfection of treatment	Hydric support (liters)	Dehydration	Time (h) postinfection of:	
		Start	Duration				Death	Slaughter
1 (M)	5.5	24	32	24, 32, 41, 46, 53	2.5	−		72
2 (M)	5.5	17		None	2.5	+	47	
3 (M)	8	12		None	2.5	+	51	
4 (F)	5.5	18	47	18, 24, 36, 48, 54	2.5	−		72
5 (M)	6	18	30	18, 35, 45, 51, 57	2.5	−		72
6 (F)	5.5	18		None	6	+	48	
7 (M)	5	12	36	13, 25, 36	1.5	−		72
8 (M)	7	21	27	22, 34, 46	1.5	−		72
9 (M)	6	16	33	16, 26, 40, 56	2	−		72
10 (F)	2	18		None	8	+	41	
11 (M)	5	21	27	21, 36, 48, 58	2	−		72
12 (M)	5	23	33	23, 28, 32, 38, 48, 63	3	−		72
13 (M)	5.5	21		27, 29, 33,	1.5	+	48	
14 (F)	5	15	57	15, 25, 37, 49, 61	2.5	−		72
15 (M)	8	18		None	8	+	47	
16 (M)	8	14	48	14, 26, 38, 60	1	−		72
17 (M)	6	17		None	7	+	48	
18 (F)	6	13.5	52.5	14, 24, 38, 47, 59	2.5	−		72
19 (F)	5	13	57	13, 25, 37, 49, 54	2.3	−		72
20 (M)[b]	8	17		17, 24, 42	4.2	+	45	
21 (F)[b]	7	13		13, 22, 37	4.2	+	44	
22 (F)[b]	6	14		14, 22, 30, 39, 47	9	+	62	
23 (M)	4	19		None	6	+	52	
24 (M)[c]	4	22		22, 32, 37	4	+	41	
25 (F)[d]	6	14		14, 22, 28, 37, 47	11	+	62	
26 (M)[e]	6	12	30	12, 27, 42, 67	6.7	−		72

[a] Calves (M, male; F, female; IgG free and germfree) received from 1.02×10^{10} to 1.6×10^{10} ETEC organisms. Calf 13 received antiadhesins 5 to 6 h after the onset of diarrhea. Calves 1 to 11 received antiadhesin preparation p1, and calves 12 to 19 received p2. Rotaviruses were detected in calves 2, 16, 18, and 19.
[b] Preparations containing amino acids (6.7 g), salts (1.80 g), free sugars (110 mg), and water (2.8 liters) were administered two or three times daily.
[c] An oral rehydratant containing glucose (21.75 g), salts (12.8 g), and water (1.5 liters) was given three times daily.
[d] Calf 25 received a complementary diet (amino acids [10 g], salts [6.1 g], glucose [10 g], and water [2 liters] twice daily).
[e] Calf 26 was treated with antibiotics.

(0.5 and 5 mg of amino acid equivalents per ml, respectively), whereas the antiadhesin preparations inhibited MRHA by 50 ± 3 and 96 ± 4%. Furthermore, the calves treated with these hydrolysates died with symptoms of diarrhea within 62 h of bacterial inoculation. No corresponding drop in the number of *E. coli* that adhered to mucosa was observed (Table 3).

Administration of antiadhesin preparations led to a fall in the number of adherent bacteria in the three parts of the small intestines. The numbers of challenge bacteria that adhered to the mucosa (expressed as $\log_{10}$ numbers of bacteria per gram of epithelial tissue) were similar in calves treated with p1 (calves 1, 4, 5, and 7 to 11) and those treated with p2 (calves 12 to 14, 16, 18, and 19) (Table 3). In contrast, there were significant differences between the means of the control group (12 untreated or orally fluid-treated calves) and the test group (13 calves) (1.71 $\log_{10}$ bacteria for the duodenum, 2.72 $\log_{10}$ bacteria for the jejunum, and 1.82 $\log_{10}$ bacteria for the distal part of the ileum; $P < 0.01$).

The glycoprotein glycan hydrolysates, under the conditions described in Materials and Methods, inhibited adhesion of *E. coli* K99 to the cells of intestinal villi in treated calves. A minimum amount of 0.5 mg of glycans inhibited the adhesion of 10^8 bacteria.

Elimination of ETEC in the lumen. Since administration of glycoprotein glycans appeared to interfere with adherence of *E. coli* K99$^+$ ST$^+$ to intestinal epithelium, we wanted to find out whether bacteria were in fact eliminated by this treatment. We therefore compared the free bacterial population in the lumen with that adhering to the mucosa. The ratio of free bacteria to adherent bacteria (Table 4) was high in the jejunum of a cured calf (calf 12). However, this ratio was considerably reduced in a calf with a partial cure (calf 14) or no cure (calf 13). Values measured in other intestinal segments (duodenum and ileum) did not seem to be correlated with clinical improvement. In an untreated calf (calf 15), the size of the adherent ETEC population was similar to that in the lumen.

Kinetics of fecal excretion of *E. coli* K99$^+$. Fecal excretion of *E. coli* could be determined by routine microbiological methods, as we used an antibiotic-resistant strain. All of the newborn calves challenged orally with 10^{10} *E. coli* K99$^+$ ST$^+$ Nal^r organisms had diarrhea. The excretion patterns are shown in Fig. 7. Pathogenic bacteria were excreted in considerable numbers for 2 to 3 days (10^8 to 10^9/g of feces) and represented the major organisms during 5 days (>90% of total flora; data not shown). All calves excreted *E. coli* K99$^+$ ST$^+$ for several weeks, but whereas the numbers of ETEC remained above 5×10^4/g of feces for more than a month in the feces of control calves, a significant decrease was observed in the feces of calves treated with the glycoprotein glycan preparations.

DISCUSSION

Diarrheal infections induced by ETEC are currently treated by agents which reduce the dehydration caused by increased excretion of water and electrolytes (31). Antibiotic therapy is often given in combination with such agents (5).

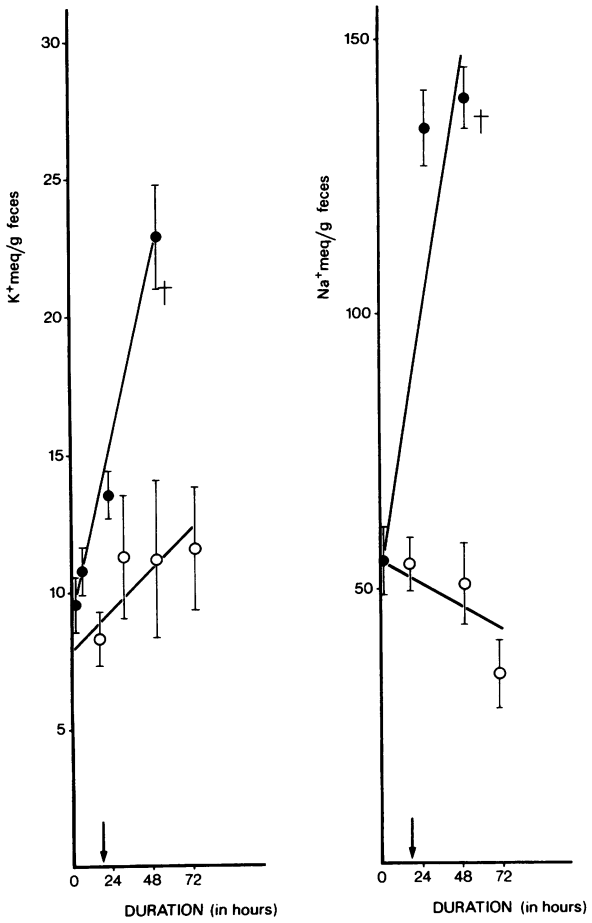

FIG. 3. Fecal potassium and sodium after challenge with ETEC. Time zero corresponds to infection. Symbols: ●, control calves; ○, treated calves; ↓, start of antiadhesin treatment; †, death. The mean ± the standard deviation of the mean is given for four control calves and seven treated calves.

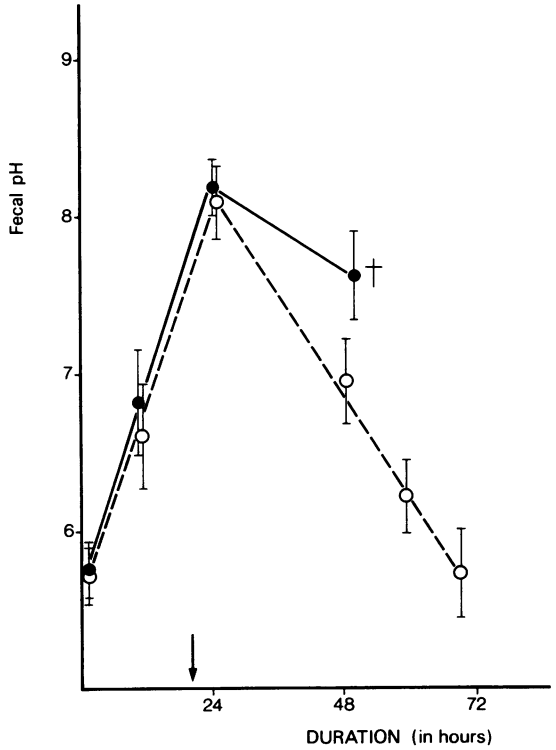

FIG. 4. pH of feces in calves challenged with ETEC. Symbols: ●, control calves; ○, treated calves; ↓, start of antiadhesin treatment; †, death. The mean ± the standard deviation of the mean is given for 12 controls and 13 treated calves. Infection was at time zero.

Extensive use of antibiotics has contributed to the development of antibiotic-resistant *E. coli* strains that infect cattle and other animal species (24, 26). Considerable interest has therefore been shown in immunoprophylaxis against ETEC pili. Pregnant cows may be protected by vaccination and transmission of specific anti-pilus antibodies by colostrum (7, 13). In some cases, an immunoserocolostrum has been administered (35).

These vaccines and, in general, any agents that affect ETEC adhesion to intestinal mucosa cells reduce the pathogenicity of microorganisms. Among the various substances evaluated in vitro, recent studies have indicated the value of antibiotics at subinhibitory doses (12, 22) or hydrophobic compounds, such as aromatic hydrocarbons (16). Increased understanding of the virulence of ETEC and other pathogenic agents at the molecular level has led to studies of analogs either of pili and other structures present on infectious agents or of receptors recognized by these structures.

Our results show that experimentally infected animals can be successfully treated by agents which act on the attachment sites of bacterial pili. These nontoxic molecules, obtained from plasma of normal cattle, mimic the glycan moieties of natural receptors. In vitro assays showed that these receptor analogs do not act via an antibody-antigen-type reaction but directly interfere with the attachment process.

The sialoconjugates of adult bovine plasma from which the glycoprotein glycans used in these experiments were ob-

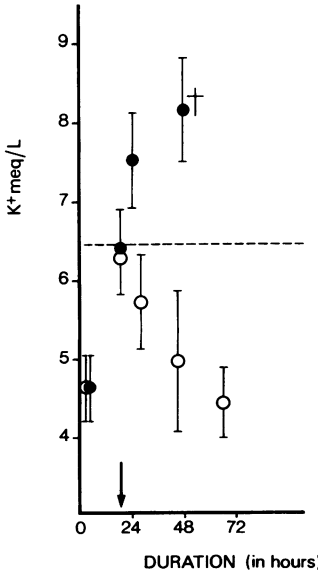

FIG. 5. Potassium levels in plasma of calves challenged with ETEC. Symbols: ●, control calves; ○, treated calves; ↓, start of antiadhesin treatment; †, death; ----, upper limit of normal values. The mean ± the standard deviation of the mean is given for four controls and seven treated calves. Infection was at time zero.

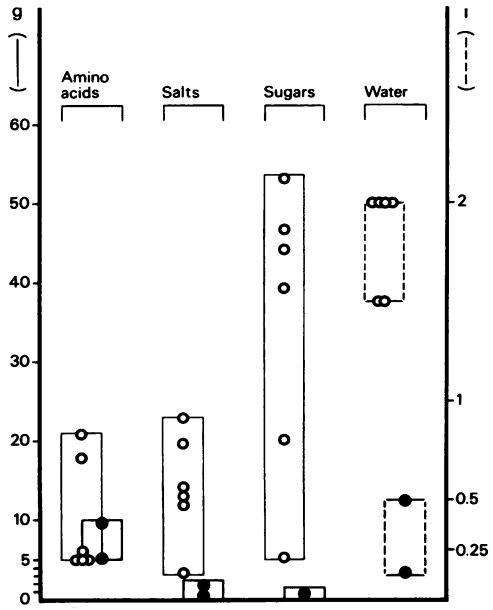

FIG. 6. Contents of commercial oral rehydratants and antiadhesin preparations. Six rehydratant compositions (○) were compared with antiadhesin preparations (●) on the basis of amino acid, sugar, salt (in grams), and fluid (in liters) contents.

TABLE 4. Ratio of free ETEC in the lumen to adherent ETEC on intestinal epithelium[a]

Calf	Free/adherent ETEC ratio in:		
	Duodenum	Jejunum	Ileum
Completely cured (no. 12)	13	2,706	166
Partially cured (no. 14)	7	160	156
Noncured (no. 13)	13	8	101
Control (no. 15)	2	12	5

[a] All calves were inoculated with 10^{10} K99$^+$ ST$^+$ Nal^r ETEC organisms. The small intestines were carefully unfolded, ensuring that the contents were undisturbed. The ratio was determined from the number of bacteria that formed colonies on Minca medium containing nalidixic acid.

tained bear terminal NeuGc sequences (8) which are not present in IgG glycans (30). The biantennary glycan structure of bovine transferrin (present in fraction II), which contains only the NeuAc(α2-6)Gal sequence (39), had no inhibitory action. However, the non-immune system *N*-glycosylated glycoproteins (8, 9) bearing the minimal sequence NeuGc(α2-3)Gal of the receptor site (Fig. 2) did inhibit adhesion of K99$^+$ *E. coli* organisms. This is in agreement with the established structures of the K99 pilus receptor (4, 17, 23, 33, 36). It should be noted that the glycoprotein glycans in fraction III may be partially masked by protein structures. Hydrolysis would thus tend to reveal their activity.

The in vivo data were consistent with the in vitro results. Nevertheless, we could not establish with confidence that

TABLE 3. Concentrations of adherent ETEC organisms in different segments of calf small intestines

Calf no.	Log_{10} no. of bacteria/g in small intestine wall scrapings[a]					
	Duodenum		Jejunum		Ileum	
	Treated calves	Controls	Treated calves	Controls	Treated calves	Controls
1	5.93		6.88		7.26	
2		8.04		9.94		0.94
3		6.77		9.97		9.61
4	6.07		6.68		6.00	
5	4.64		5.25		6.90	
6		6.27		7.84		9.54
7	3.47		4.34		6.04	
8	5.17		4.39		5.49	
9	5.04		6.32		6.54	
10		6.77		8.54		7.47
11	3.34		5.25		6.00	
12	5.73		4.90		7.30	
13	6.44		9.17		8.95	
14	7.23		9.34		8.95	
15		7.27		8.00		9.47
16	5.59		7.14		7.20	
17		7.96		9.27		9.22
18	4.85		7.47		7.60	
19	5.27		6.30		6.60	
20		6.53		9.83		9.47
21		6.75		8.84		8.57
22		7.58		9.84		9.36
23		6.89		9.38		9.23
24		6.34		8.95		6.78
25		6.90		9.43		7.47
Mean (SD); variance[b]	5.29 (1.08); 1.18	7.00 (0.58); 0.34	6.42 (1.62); 2.62	9.15 (0.73); 0.54	6.98 (1.07); 1.14	8.84 (1.03); 1.06

[a] Resistance to nalidixic acid was used as a marker of pathogenicity. Pathogenic flora represented 93.5 to 99.1% of the total counted bacterial populations. Results were analyzed by Statview (Brain Power, Calabasas, Calif.). *P* was 0.005 for adherent bacteria compared with controls. Populations in antibiotic-treated calf intestine were 3.90 log_{10} bacteria in the duodenum, 6.55 log_{10} bacteria in the jejunum, and 5.60 log_{10} bacteria in the ileum.

[b] For duodenal, jejunal, and ileal scrapings, the differences between treated and control calves were 1.71 log_{10}, 2.72 log_{10}, and 1.82 log_{10}, respectively.

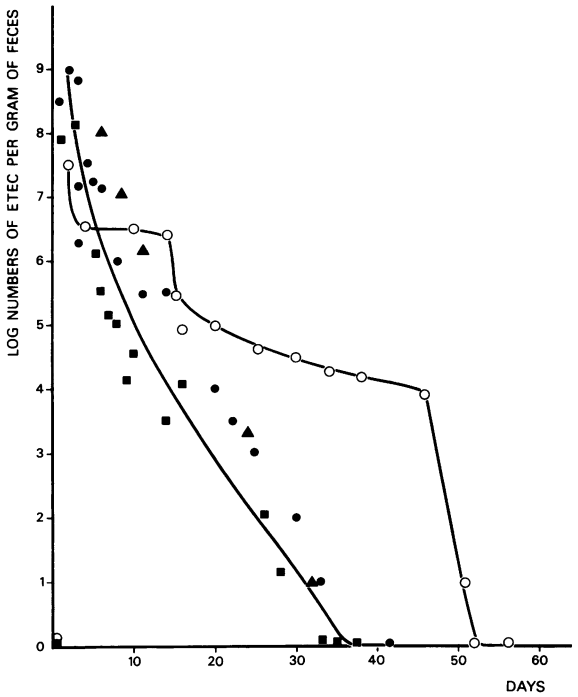

FIG. 7. Excretion pattern of the challenge strain by four calves after experimental infection with 10^{10} ETEC organisms. Two calves fed with 2 liters of colostrum (▲ and ■) and one colostrum-deprived calf (●) were treated with antiadhesin preparation p1 (250 mg of glycans twice daily) during diarrhea and after cure. One newborn calf that received colostrum was challenged at 12 h (○). Log_{10} numbers of bacteria per gram of feces are shown.

these receptor analogs reduced bacterial adherence in vivo, since the bacteria were counted at different times in the treated and control groups. However, the difference in the adherent populations of *E. coli* was more likely to have been the result of their antiadhesive properties than of fluid replacement or a nutritional effect. Although we could not rule out an action of the hydrolysate peptidic moiety, destruction of the glycan structure led to loss of biological activity. This shows at least that the peptide moiety is not sufficient for a therapeutic effect.

To account for our results, we assumed that the glycan conjugates enhance removal of bacteria from epithelial cells or the mucus layer. Although analyses of bacterial elimination into the lumen or feces were carried out on only a small number of animals, the reduction in bacterial adherence to the intestinal wall appeared to be related to enhanced elimination of the bacteria. Binding of bacteria to intestinal cells is thus affected by binding of the bacteria to exogenous glycans. Adhesion of *E. coli* K99, which occurs via a mechanism of positive cooperativity at high binding site densities, is modified in the presence of receptor analogs. Recognition of sites is reduced, and negative cooperativity occurs (28).

In the experiments reported here, the effective doses that provided a cure in less than 72 h ranged from 1.5 to 3 g per calf. These differences were due, at least in part, to differences in the balance between bacteria and the quantity and quality of intestinal mucus and cell receptors (M. Mouricout and R. Julien, Proceedings of the IX International Symposium on Glycoconjugates, Lille, France, abstr. no. G14, 1987). The terminal sialic acid moiety is important for recognition, and individual differences in sensitivity to colibacillosis may be due to differences in the NeuAc and/or NeuGc contents of mucosal receptors.

It is of interest that there is considerable variability in the substitution of sialic acid residues in different bovine tissues which appears to be age related (8). Furthermore, treatment with glycoprotein glycans appeared to remove attached bacteria in the jejunum preferentially. This may be due to differences in the nature or content of receptors in the three parts of the small intestine. In newborn calves, sialylated glycosphingolipids are more abundant in the jejunum than in other segments. The ganglioside pattern also varies along the intestinal tract; disialogangliosides that predominate in the jejunum may form receptor structures that are recognized preferentially by K99 pili (M. Mouricout, M. Milhavet, E. Boirleaud, B. Quintard, and R. Julien, Japanese-German Symposium on Sialic Acids, Berlin, Federal Republic of Germany, abstr. no. D2.24, 1988).

The curative effect of the glycans was observed in a situation in which host defense mechanisms were not yet operative. The newborn calves used were colostrum deprived and thus did not benefit from maternal immunoglobulins. Our finding that relatively small amounts of glycan hydrolysates exerted a protective action in heavily infected calves is in agreement with the results of Otnæss et al., who showed that nonimmunological components of milk, even in trace amounts, may protect against certain gastrointestinal infections in rabbits (33).

These natural receptor analogs could be digested, but calf intestine appears to have only weak glycolytic activity. Amylase activity is detected, but there is no oligoglucosidase (6). Although we have no direct evidence of the evolution of these receptor analogs in the digestive tract, preliminary studies using radiolabeled analogs indicated the absence of degradation in unweaned mice (unpublished data).

Treatment of ETEC diarrhea in neonatal calves by bacterial receptor analogs provides a new and promising approach to early therapy, either alone or in combination with other treatments. Plasma-derived glycoprotein glycans of the *N*-acetyllactosamine type (Fig. 2) contain sugar sequences which may be the major determinants of the recognition sites in the digestive tract for various bacterial lectins (9). Bacterial adhesins (pili, flagella, and external membrane proteins) are known to have specific affinities for a variety of glycan structures (26a, 29, 32). Clinical studies of farm animals have demonstrated the effectiveness of extracts against strains of *Campylobacter* sp. or *Streptococcus* sp. in palliating the effects of infection with these bacteria (M. Mouricout, J. M. Petit, T. Chibois, G. Dubost, C. Gayaud, F. Noel, R. Shrivastava, R. Julien, and J. A. Nicolas, Symposium Ecologie Microbienne du Tube Digestif chez l'Homme et les Animaux, Paris, France, abstr. no. 7, 1985).

These analogs are simple to use (oral administration) and are derived from a cheap raw material (blood plasma). They could therefore be used in other animal species, as well as humans, in situations in which bacterial colonization and adhesion depend on the recognition of specific glycans in the digestive tract.

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