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# Isolation and Structural Characterization of the Equine Erythrocyte Receptor for Enterotoxigenic *Escherichia coli* K99 Fimbrial Adhesin

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**The erythrocyte receptor for *Escherichia coli* K99 fimbrial adhesin was isolated from equine erythrocytes and characterized as Neu5Gc-α(2→3)-Galp-β(1→4)-GLcp-β(1→1)-Ceramide. This glycolipid acted as the receptor for K99 by four different experimental approaches: inhibition of equine erythrocyte hemagglutination by preincubation of K99-positive bacteria or purified K99 fimbriae with the isolated glycolipid; inhibition of attachment of K99-positive bacteria to porcine intestinal epithelial cells in the presence of the isolated glycolipid; induction of binding of K99-positive bacteria or purified K99 fimbriae to normally unreactive guinea pig erythrocytes by coating these cells with the isolated glycolipid; and isolation of the receptor by affinity chromatography with K99 coupled to CNBr-activated Sepharose 4B, indicating a strong interaction between K99 and the isolated glycolipid.**

Noninvasive enterotoxigenic *Escherichia coli* strains possessing the K99 antigen are known to cause diarrhea in neonatal calves, lambs, and piglets (9). The pathogenicity of these strains is mainly based on two virulence factors: the ability to colonize the small intestine and the production of enterotoxins which cause the actual diarrhea. The surface antigen K99 is involved in the adherence of the bacteria to the brush border of epithelial cells in the small intestine of susceptible animals. The antigen has been characterized as a fimbria-like structure composed of a single repeating protein subunit with a molecular weight of 18,500 (3). Purified K99 antigen causes a strong, non-mannose-sensitive, hemagglutination of horse erythrocytes, a weaker reaction with sheep erythrocytes, and no activity with guinea pig erythrocytes (9). K99-positive cells adhere to intestinal epithelial cells of pigs and calves (24), and they exhibit a strong, non-mannose-sensitive hemagglutination of horse and sheep erythrocytes. The interaction of K99 with the epithelial cells and erythrocytes indicates the presence of a receptor substance in the membrane of these cells. Little is known about the nature and structure of this receptor. Based on hemagglutination experiments, Faris et al. suggested a $GM_2$-like substance as the K99 receptor (RK99) (6). It was also reported that K99 had affinity for terminal *N*-acetylgalactosamine and sialic acid residues of complex glycoconjugates. (M. Lindahl and T. Wadström, Proc. 7th Int. Symp. Glycoconjugates, Lund, Sweden, p. 635, 1983). However, direct evidence for the involvement of these structures in K99 binding in vivo can only be provided by the isolation of RK99(s) from erythrocytes or epithelial cells to which K99-positive bacteria adhere. The aim of this study was to isolate and characterize the receptor that is involved in the K99-mediated hemagglutination of equine erythrocytes.

## MATERIALS AND METHODS

**Bacterial strains and media.** The K99-producing *E. coli* F18 (O101:K$^-$:K99) was used in hemagglutination and adhesion experiments. Purified K99 adhesin was prepared from *E. coli* F82 (O101:K$^-$:K99) harboring plasmid pRI 9906-1 (28). Minca medium supplemented with yeast extract (1 mg/ml; Oxoid Ltd., London, England) (11) was used for cultivation of K99-producing bacteria. For the cultivation of *E. coli* F82, 100 μg of ampicillin per ml was added to the medium

**Isolation of the equine erythrocyte RK99.** Erythrocytes were harvested from freshly drawn, heparinized blood from horses and were washed three times with 0.9% NaCl at 4°C. Subsequently, the packed erythrocytes were freeze-dried. The freeze-dried equine erythrocytes were extracted continuously with chloroform-methanol (2:1 [vol/vol]) in a Soxhlet apparatus for 24 h, followed by extraction with chloroform-methanol (1:9 [vol/vol]) for another 24 h (Fig. 1). The total lipid extract (CM extract) was subjected to mild alkaline hydrolysis, acidification, and partition as previously described (19). After partition, the concentrated chloroform phase was loaded on a silicic acid column (particle size, 0.2 to 0.5 mm; 30-70 mesh; E. Merck AG, Darmstadt, Federal Republic of Germany). Lipids were eluted with chloroform, methanol-chloroform (1:9 [vol/vol]), methanol-chloroform (3:1 [vol/vol]), and methanol (Fig. 1). The load was 10 mg of lipid material per g of silicic acid, and the elution volume was 10 ml/g of silicic acid. Lipids were isolated after separation by preparative thin-layer chromatography (TLC) on plates (Silica Gel 40; 20 by 20 cm; layer thickness, 0.25 mm; Merck) in the solvent system chloroform-methanol-water (65:25:4 [vol/vol/vol]). The lipids were extracted from the silica gel with the developing solvent (two times), chloroform-methanol (1:1 [vol/vol]), and with methanol. All steps in the isolation of RK99 were monitored by high-pressure TLC (Silica Gel 60, for nano-TLC; Merck).

**Isolation of the erythrocyte RK99 by affinity chromatography.** An alternative method was developed to isolate RK99 by affinity chromatography. The lipid fraction obtained after alkaline hydrolysis and partition was used as starting material. K99 fimbriae were isolated and purified as described by de Graaf et al. (3). Purified K99 fimbriae were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by incubating 10 mg of K99

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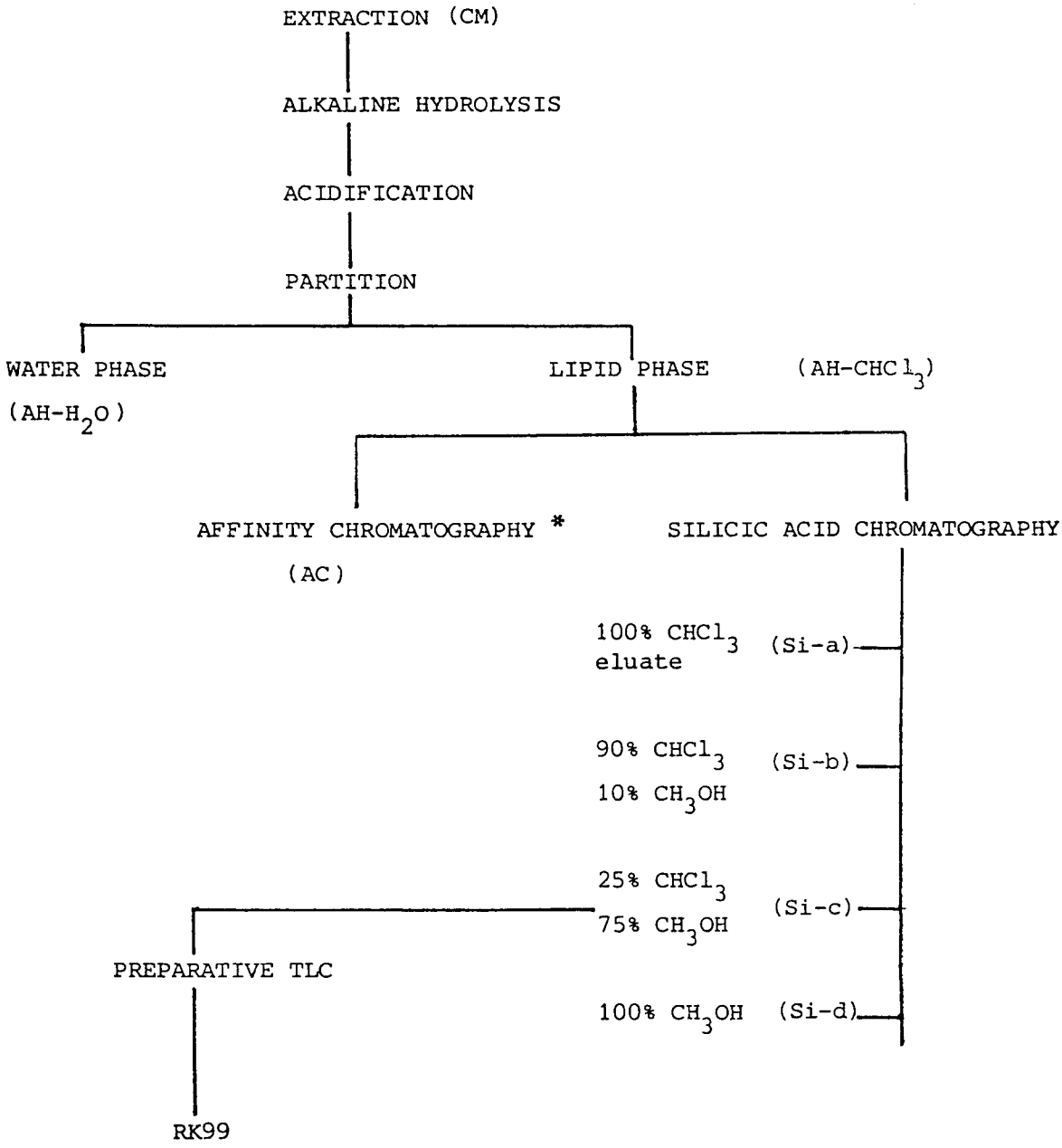


FIG. 1. Isolation scheme for equine erythrocyte RK99. (*) Alternative way to isolate the receptor by affinity chromatography with K99 coupled to CNBr-activated Sepharose 4B.

protein for 2 h at room temperature with 3 g of activated Sepharose. Unbound material was washed away, any remaining active groups were blocked, and noncovalently absorbed protein was removed as described by the manufacturer. Subsequently, 30 mg of the starting lipid material was incubated with K99-Sepharose in PBSM buffer (phosphate-buffered saline, 10% methanol) in a total volume of 8 ml during 2 h at room temperature. To remove unbound lipid material, the gel was washed five times with PBSM buffer and two times with methanol. Bound lipid material was removed from the gel by repeated extraction with chloroform-methanol (2:1 [vol/vol]).

**Hemagglutination inhibition test with whole cells of *E. coli*.** From an overnight culture of *E. coli* cells, portions of 0.3 ml were centrifuged. The pellets were suspended in 0.25 ml of KTM buffer (0.1 M Tris [pH 7.4] containing NaCl [7.5 g], KCl [0.383 g], $MgSO_4 \cdot 7H_2O$ [0.318 g], $CaCl_2 \cdot 2H_2O$ [0.404 g], and mannose [5 g/liter]) (4) (control) or in KTM buffer with various amounts of the lipid fraction to be tested. The suspensions were incubated for 30 min at 37°C. Then the cells were spun down to remove unbound lipids, and the pellets were resuspended in 0.125 ml of KTM buffer. Serial twofold dilutions of these suspensions were made in KTM buffer with polystyrene trays containing V-shaped cups (Cooke microtiter system; Sterilin, Teddington, Middlesex, England). The volume in each cup was 50 μl. Finally, 50 μl of a 1% erythrocyte suspension in KTM buffer was added to each cup. The hemagglutination titer was defined as being

the highest dilution of bacteria that still caused obvious hemagglutination after incubation for 2 h at 4°C.

**Hemagglutination inhibition test with purified K99 fimbriae.** Serial twofold dilutions of purified K99 fimbriae were made in KTM buffer and in KTM buffer with various amounts of a lipid fraction with polystyrene trays containing V-shaped cups. The volume in each cup was 50 μl. Subsequently, 50 μl of a 1% erythrocyte suspension in KTM buffer was added to each cup. After 2 h of incubation at 4°C, the hemagglutination titer was read.

**Adhesion inhibition test.** Epithelial cells were prepared from the small intestine of pigs as described by Sellwood et al. (25). The adhesive capacity of *E. coli* F18 in the presence of 1% D-mannose was determined by phase-contrast microscopy as described by Svanborg Edén et al. (27). The ability of erythrocyte RK99 to inhibit attachment was tested by preincubation of bacteria with various amounts of RK99 in KTM buffer for 60 min at 37°C, followed by the addition of epithelial cells.

**Coating of guinea pig erythrocytes with RK99.** A 20% guinea pig erythrocyte suspension in 0.9% NaCl (50 μl) was mixed with various amounts of RK99 suspension in KTM buffer without D-mannose (500 μl) and was incubated with gentle shaking for 3 h at 37°C. After being washed three times in KTM buffer to eliminate unbound receptor, the erythrocytes were suspended in the same buffer to a final concentration of 3% for hemagglutination tests.

**Methods for structural analysis. (i) Sugar analysis.** RK99 (1 mg) was dissolved in 1 M methanolic HCl (0.5 ml) and heated for 24 h at 85°C. Then the solution was extracted three times with *n*-hexane. After neutralization of the methanol phase with silver carbonate, the mixture of sugar methyl glycosides was treated with acetic anhydride and trimethylsilylation


FIG. 2. High-pressure TLC of the various lipid fractions isolated from equine erythrocytes. Lanes: A, CM fraction; B, AH-$H_2O$; C, AH-$CHCl_3$; D, Si-a; E, Si-b; F, Si-c; G, Si-d; H, RK99; and I, AC. Load: lanes A to G, 20 μg; lane H, 4 μg; and lane I, 10 μg. L, hemolytic compound; R, RK99; g, glycolipid. The chromatogram was developed in chloroform-methanol-water (62:25:4). Anisaldehyde was used as the reagent for detection of RK99 (27). For the specific detection of glycolipids, a separate chromatogram was sprayed with 2% α-naphthol in ethanol and with concentrated $H_2SO_4$.

TABLE 1. Hemagglutination inhibition and yield of the various lipid fractions isolated from equine erythrocytes

| Fraction | MIC (μg/ml)[a] | Yield (% wt)[b] |
|---|---|---|
| CM | 12,000 | 100 |
| AH-$H_2O$ | 7,100 | Not determined |
| AH-$CHCl_3$ | 750 | 59 |
| Si-a | 12,000 | 33 |
| Si-b | 280 | 3 |
| Si-c | 33 | 14 |
| Si-d | 2,600 | 5 |
| RK99 | 7 | 3 |
| AC | 30 | 2 |

[a] MIC is defined as the minimal concentration of K99-positive *E. coli* cells inhibiting equine erythrocyte hemagglutination.

[b] The amount of lipids in each fraction was determined by weight after evaporation of the solvent under reduced pressure and was related to the amount of lipid in the chloroform-methanol extract (CM fraction). Yield (% weight) = lipid yield in fraction (mg)/lipid yield in CM extract (mg) × 100%.

reagents and was analyzed by capillary gas-liquid chromatography on CPsil5 as described previously (16, 19).

**(ii) Fatty acid analysis.** The hexane phase (see above) was washed with 0.1 M HCl and, after concentration, was analyzed for fatty acid methyl esters by capillary gas-liquid chromatography on a 25-m CPsil5 WCOT glass capillary column (temperature range, 100 to 230°C at 6°C/min).

**(iii) Permethylation.** RK99 (1.5 mg) was permethylated with sodium methylsulfinylmethanide in dimethyl sulfoxide-methyl iodine by the method of Hakomori (12, 13). The permethylated product obtained by chloroform extraction was purified on a column (3 by 0.5 cm) of Kieselgel 60 (Merck); after applying the material, the column was washed with chloroform (10 ml), and subsequently permethylated RK99 was desorbed with 2% methanol in chloroform (10 ml).

**(iv) Partially methylated alditol acetates.** A sample of the purified permethylated RK99 (0.2 mg) was subjected to hydrolysis with 0.25 M $H_2SO_4$ in 90% acetic acid (0.3 ml). After 4 h at 85°C, the hydrolysis mixture was applied to a column of Bio-Rad AG-3 (3 by 0.5 cm; acetate form). The column was washed with 15 ml of methanol, and the combined eluate was evaporated to dryness. The residue was reduced with $NaB^2H_4$ and acetylated with acetic anhydride in pyridine (1:1 [vol/vol]) as previously reported (13).

**(v) Exoglycosidase incubations.** For monosaccharide sequence analysis, RK99 (1 mg) was incubated with α-sialidase from *Clostridium perfringens* (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (1), and the asialo-RK99 obtained was incubated with β-galactosidase (Boehringer Mannheim) from *E. coli* (8). The digestions were followed by TLC with the solvent system mentioned above.

**(vi) Gas-liquid chromatography–mass spectrometry.** Direct insertion mass spectrometry of permethylated RK99 was carried out on an AEI MS-902 apparatus with an ion source temperature of 230°C, an accelerating voltage of 4 kV, an electron energy of 70 eV, and an ionizing current of 100 μA. Gas-liquid chromatography of neutral, partially methylated alditol acetates on 3% OV-225 at 185°C was carried out as previously described (13, 15). Combined gas-liquid chromatography–mass spectrometry of these derivatives was performed on a Carlo Erba GC/Kratos MS80/Kratos DS55 system (electron energy, 70 eV; accelerating voltage, 2.7 kV; ionizing current, 100 μA; ion source temperature, 225°C; column, 3% OV-225 on Chromosorb WHP, 100-120 mesh; oven temperature, 185°C).

$^1$H-nuclear magnetic resonance spectroscopy (500 MHz)

TABLE 2. Effect of RK99 and AC fractions on K99-mediated hemagglutination of equine erythrocytes

| Fraction | Concn added (μg/ml) | HA titer[a] |
|---|---|---|
| | | 128 |
| RK99 | 300 | 0 |
| RK99 | 20 | 32 |
| AC | 300 | 8 |

[a] Hemagglutination (HA) was determined with a serial twofold dilution of purified K99 fimbrial protein with an initial concentration of 0.25 mg/ml.

of RK99 was performed with a Bruker WM-500 spectrometer (SON Facility, Department of Biophysics, Nijmegen University, The Netherlands) operating in the Fourier transform mode at a probe temperature of 27°C. Before analysis, RK99 (2 mg) was repeatedly treated with a $^2H_2O$-$CH_3O^2H$ mixture, applying intermediate lyophilization. The resulting residue was dissolved in dimethyl sulfoxide-$^2H_6$-$^2H_2O$ (98:2 [vol/vol]) containing tetramethylsilane as internal standard (2, 20). Resolution enhancement of the spectrum was achieved by Lorentzian-to-Gaussian transformation (5).

## RESULTS

**Isolation of the erythrocyte RK99.** The isolation scheme for the erythrocyte RK99 is depicted in Fig. 1. The various fractions were analyzed with TLC (Fig. 2), and the receptor activity of each fraction was determined by the hemagglutination inhibition test with whole *E. coli* F18 cells. MICs, specific activity, and the yield of each fraction as a percentage of CM extract are presented in Table 1.

After the extraction of 20 g of freeze-dried equine erythrocytes, ca. 400 mg of brown-colored CM extract was obtained with minor receptor activity (Fig. 2, lane A; Table 1). Some receptor activity was observed in the water phase fraction (designated as AH-$H_2O$) obtained after alkaline hydrolysis, acidification, and partition of the CM extract (Table 1). This fraction, however, was not further investigated since TLC did not reveal the presence of any lipid compounds (Fig. 2, lane B). The lipid phase fraction (designated as AH-$CHCl_3$) obtained after partition showed a significant increase in receptor activity with respect to the CM extract (Table 1). After TLC, glycolipid staining with α-naphthol reagent indicated the presence of four glycolipids in the AH-$CHCL_3$ fraction: a major glycolipid with an $R_f$ value of 0.17 and three minor glycolipids with $R_f$ values of 0.43, 0.47, and 0.61 (designated as g in Fig. 2, lane D). The compounds of the AH-$CHCl_3$ fraction were further separated by silicic acid column chromatography. The lipids eluted with chloroform (Si-a) showed minor receptor activity. Fraction Si-b (Fig. 2, lane E) showed some receptor activity, but receptor activity in the Si-c fraction (Fig. 2, lane F) was much higher, indicating the presence of the majority of RK99 in this fraction. The major glycolipid at an $R_f$ value of 0.17 and the glycolipid at an $R_f$ value of 0.61 could be detected in this fraction. The Si-d fraction (Fig. 2, lane G) did not show significant receptor activity. The compound in this fraction with the same $R_f$ value as the main glycolipid in lanes C and F did not react with the α-naphthol reagent and therefore is most probably not glycolipid. To trace RK99, preparative TLC was performed with the Si-c fraction, and different parts of the chromatogram were scraped off and tested for receptor activity. This procedure revealed that only one compound was responsible for the receptor activity being the major glycolipid at an $R_f$ value of 0.17 (designated as R in Fig. 2, lane C). The isolated receptor (RK99) showed high activity (Table 1), and its purity was tested with TLC (Fig. 2, lane H).

TABLE 3. Inhibition of attachment of K99-positive *E. coli* to intestinal epithelial cells of pigs by the RK99 glycolipid

| RK99 added (μM)[a] | Mean no. of bacteria/cell[b] |
|---|---|
| 0 | 23 ± 7 |
| 20 | 7 ± 6 |
| 40 | 2 ± 2 |
| 80 | 0 |

[a] The concentration of RK99 is based on an average molecular weight of 1,251.

[b] These values were obtained after investigation by phase-contrast microscopy of 40 epithelial cells for each incubation.

With affinity chromatography, a lipid fraction (designated as AC; Fig. 1) could be extracted that showed high receptor activity (Table 1). Analysis on TLC (Fig. 2, lane I) revealed that the main lipid compound of this fraction was identical with the RK99 fraction that was isolated by silicic acid column chromatography and preparative TLC. To establish that the receptor activity (Table 1) was the result of an interaction between RK99 and K99 fimbriae and not due to an Si-a-specific interaction between the RK99 glycolipid and some other bacterial cell wall component, the effect of RK99 and lipid fraction AC on the hemagglutination mediated by purified K99 fimbriae was also tested. As a consequence of hemolytic activity, this test was not possible with the CM, AH-$CHCl_3$ and Si-c fractions, since in contrast to whole bacteria, purified K99 fimbriae could not be separated from the hemolytic compound by centrifugation. The hemolytic compound (designated as L; Fig. 2, lane C) was traced after preparative TLC. This compound gave hemolysis of equine erythrocytes at a concentration of 10 μg/ml. The fractions RK99 and AC did not contain the hemolytic compound L. Strong inhibition was observed with RK99 (Table 2). The AC fraction also showed considerable receptor activity, but as a consequence of especially non-lipid impurities (as indicated by the incomplete solubility of this fraction in chloroform), a ca. fourfold greater amount of AC was required for the same inhibitory effect as was observed with RK99 (Table 1 and 2). These results indicate that the isolated glycolipid acts as a receptor for K99 fimbriae.

**Inhibition of the adherence of K99-positive *E. coli* cells to porcine intestinal epithelial cells.** The adhesion of *E. coli* F18 cells to porcine intestinal epithelial cells could be inhibited by small amounts of equine erythrocyte RK99 (Table 3). This inhibition suggested that the added erythrocyte receptor competed with the natural intestinal receptor for K99, indicating a possible structural relationship between erythrocyte and intestinal receptors.

**Agglutination of guinea pig erythrocytes coated with RK99.** Guinea pig erythrocytes are normally not agglutinable by *E. coli* strains possessing K99 fimbriae or by purified K99 fimbriae (9). It is known that the addition of a glycolipid to a suspension of erythrocytes lacking this compound may lead to the incorporation of the glycolipid in the erythrocyte membrane. Upon incubation of a guinea pig erythrocyte suspension with various amounts of equine erythrocyte RK99, followed by washing away of unbound material, it was observed that the previously unreactive erythrocytes had become strongly agglutinable by K99-positive whole bacteria as well as by purified K99 fimbriae (Table 4). The

TABLE 4. Induction of binding between K99-positive bacteria or purified K99 fimbriae and unreactive guinea pig erythrocyte by coating with RK99 glycolipid

| Erythrocyte species | Amt of RK99 (μg)[a] | HA titer[b] with: | |
|---|---|---|---|
| | | K99-positive bacteria | K99 fimbriae |
| Horse | 0 | 128 | 1,024 |
| Guinea pig | 0 | 0 | 0 |
| Guinea pig | 100 | 64 | 128 |
| Guinea pig | 50 | 32 | 64 |
| Guinea pig | 25 | 16 | 64 |

[a] Amount of purified glycolipid receptor used for coating of guinea pig erythrocytes was as described in the text.

[b] Hemagglutination (HA) after incubation with a serial twofold dilutions of K99-positive bacteria (initial cell density corresponded to $E_{660}$ of 1.1) and purified K99 fimbriae (concentration of undiluted K99 of 1 mg/ml).

degree of agglutination increased with the amount of RK99 receptor used for coating.

**Structural analysis of RK99.** The various results presented below indicate that RK99 corresponds with the equine erythrocyte hematoside Neu5Gc-α(2→3)-Galp-β(1→4)-Glcp-β(1→1)-Ceramide (10, 29) Sugar analysis of RK99 revealed the presence of Neu5Ac, Gal, and Glc in equimolar amounts. The method employed does not determine original *N*-acyl (or *O*-acyl) substituents. This means that the native sialic acid residue may be different from Neu5Ac, as is the case for RK99 (see below).

Fatty acid analysis demonstrated the main fatty acids to be 16:0 (7.8%), 18:0 (7.1%), 18:1 (7.0%), 18:2 (3.0%), 22:0 (9.6%), 24:0 (39.4%), and 24:1 (26.1%). The mass spectrum of the permethylated RK99 is in accordance with a Neu5Gc→Hex→Hex→Ceramide sequence (18, 22). In this structure, the ceramide part is heterogeneous with respect to the fatty acid substituents of the sphingosine. Some typical fragmentations of the main component having lignoceric acid (24:0) as fatty acid are presented in Fig. 3.

Methylation analysis of permethylated RK99 by combined gas-liquid chromatography–mass spectrometry yielded equimolar amounts of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol-1-$^2H_1$ and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol-1-$^2H_1$ (13). These findings are in accordance with a 3-linked Galp unit and a 4-linked Glcp unit. Subsequent exoglycosidase digestions with α-sialidase and β-galactosidase demonstrated a sialic acid→Gal sequence in the carbohydrate chain.

The most characteristic part of the 500-MHz $^1H$-nuclear magnetic resonance spectrum of RK99 in dimethyl sulfoxide-$^2H_6$-$^2H_2O$ (98:2 [vol/vol]) is presented in Fig. 4. The occurrence of Neu5Gc as sialic acid is evident from the presence of two doublets at σ 3.893 and 3.846 ppm ($J = -16$ Hz) for the two methylene protons of the *N*-glycolyl substituent. No signals for *N*-acetyl, *O*-acetyl, or either substituent were observable. In this context, it should be noted that free Neu5Gc gives rise to only one signal for the two methylene protons of the *N*-glycolyl substituent (σ 3.870 ppm). The Neu5Gc H-3eq signal is found at σ 2.754 ppm. In the anomeric region, the signals at σ 4.189 ($J = 7.7$ Hz) and 4.153 ($J = 7.7$ Hz) correspond with β-Galp H-1 and β-Glcp H-1, respectively. The occurrence of relatively large amounts of *cis*-unsaturated fatty acids can be inferred from the intensities of the signals at σ 5.317 and 1.975 ppm, corresponding with *cis*-olefinic methine protons and methylene protons adjacent to *cis*- double bonds, respectively. In the figure, some additional assignments, based on the recently published data for Neu5Gc-α(2→3)-Galp-β(1→4)-Glcp-β(1→1)-Ceramide (20), have been included.

## DISCUSSION

In this paper, we present the isolation and structural characterization of equine erythrocyte receptor for the K99 fimbrial adhesin. The isolated glycolipid compound (RK99) was shown to act as the receptor for K99 by four different experimental approaches: inhibition of hemagglutination of equine erythrocytes by preincubation of K99-positive bacteria or purified K99 fimbriae with RK99; inhibition of attachment of K99-positive bacteria to porcine intestinal epithelial cells in the presence of the RK99 glycolipid; induction of binding of K99-positive bacteria or purified K99 fimbriae to normally unreactive guinea pig erythrocytes by coating of these cells with the RK99 glycolipid; and an alternative way to isolate the receptor by affinity chromatography with K99 coupled to CNBr-activated Sepharose 4B, indicating a strong interaction between K99 and the RK99 glycolipid. The structure of the RK99 appeared to be Neu5Gc-α(2→3)-

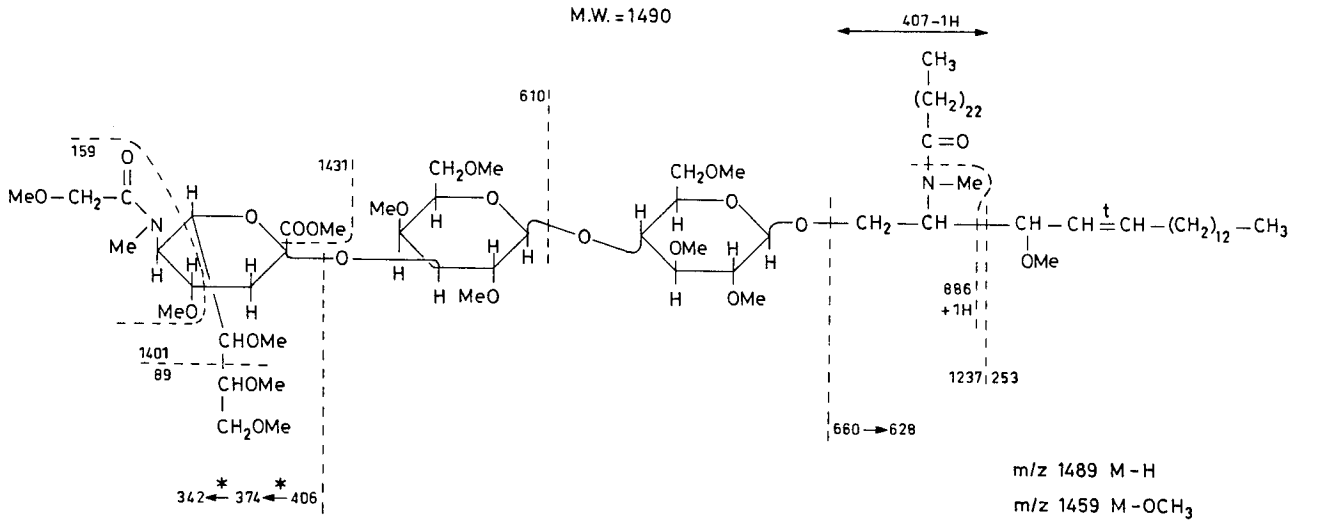


FIG. 3. Mass spectral fragmentation of permethylated Neu5Gc-α(2→3)-Galp-β(1→4)-Glcp-β(1→1)-Ceramide having lignoceric acid (24:0) as fatty acyl group.

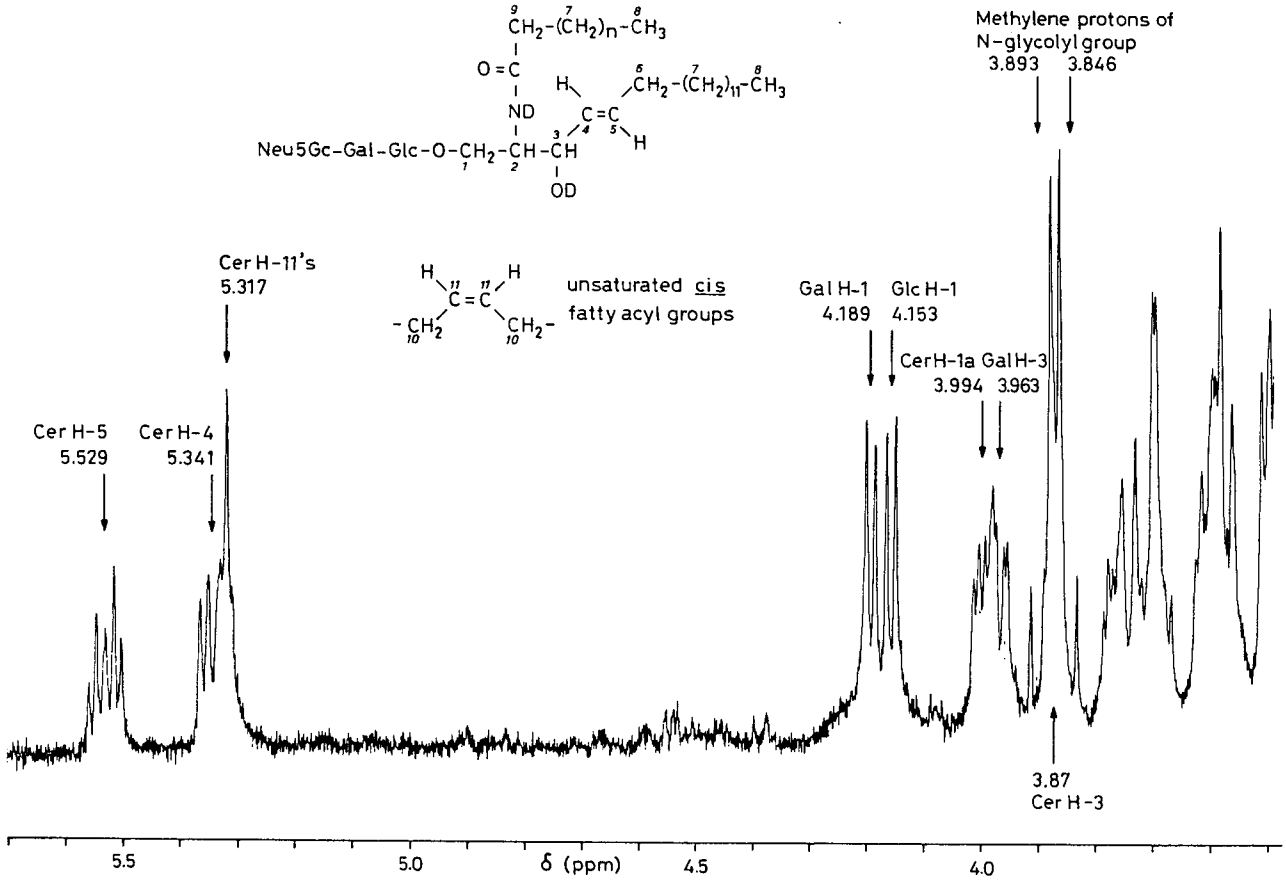


FIG. 4. Characteristic part of the 500-MHz $^1$H-nuclear magnetic resonance spectrum of Neu5Gc-α(2→3)-Galp-β(1→4)-Glcp-β(1→1)-Ceramide in dimethylsulfoxide-$^2H_6$-$^2H_2O$(98:2 [vol/vol]) at 27°C.

Galβ(1→4)-Glcβ(1→1)-Ceramide. (Fig. 3). This structure is identical to the structure of the so-called "hematoside", originally isolated from equine erythrocytes by Yamakawa et al. in 1951 (29).

Regarding the receptor structures for *E. coli* strains of different origin, at least three distinct types of cell surface carbohydrate structures are involved in fimbriae-mediated adhesion to epithelial cells. These three types of cell surface glycoconjugates are α-D-mannosyl residues for strains of *E. coli* possessing type 1 fimbriae (7), P-blood group antigens from blood group P containing the structural element α-Gal-(1→4)-β-Gal for human pyelonephritic *E. coli* strains (14), and neuraminyl α-(2→3)-galactosides for a number of human *E. coli* strains that do not interact with α-mannosyl residues or from blood group P antigens (23). In this concept, RK99 is obviously of type 3. The porcine intestinal receptor for K88-positive *E. coli* bacteria that was recently isolated (G. Nilsson and S. Svensson, Proc. 7th Int. Symp. Cyanoconjugates, Lund, Sweden, p. 637, 1983) resembles type 2 receptors since galactose residues seem to be involved in adhesion.

Faris, Lindahl, and Wadström (6) proposed a $GM_2$-like substance to be RK99 since this ganglioside, in contrast to $GM_1$ and $GM_3$, could inhibit K99-mediated hemagglutination. They suggested that the preferential inhibition by $GM_2$ is caused by the presence of a terminal GalNAc moiety in this compound. The identification of Neu5Gcα(2→3)-Galβ(1→4)Glcβ(1→1)-Ceramide as the structure of RK99 does not confirm this suggestion since no GalNAc moiety is present. Instead, based on the close structural resemblance of $GM_3$ and RK99, a strong inhibition of K99-mediated hemagglutination by $GM_3$ might be expected; although on the other hand, the *N*-glycolyl substitution in RK99 may be essential and strongly preferential to the *N*-acetyl substitution in $GM_3$.

Since K99 fimbriae mediate the adherence of the bacteria to intestinal epithelial cells in vivo, it was interesting to investigate to what extent the erythrocyte RK99 resembles the intestinal receptor of calves, lambs, or piglets. Some structural relationship is suggested by the ability of the erythrocyte receptor to inhibit adhesion of K99-positive bacteria to porcine intestinal epithelial cells (Table 3). Preliminary data obtained from an analysis of the K99-recognizing glycolipid fraction isolated from intestinal epithelial cells of calves confirm a strong structural relationship between the receptors on erythrocytes and intestinal epithelial cells. A comparable situation has been observed with pyelonephritis-associated *E. coli* strains, which recognize a similar receptor structure on both human erythrocytes and uroepithelial cells (14, 21). The isolation and characterization of the K99 intestinal receptor from calves and piglets will be continued in future experiments.

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## LITERATURE CITED


1. **Ariga, T., M. Sekine, R. K. Yu, and T. Miyatake.** 1982. Disialogangliosides in bovine adrenal medulla. J. Biol. Chem. **257**:2230–2235.
2. **Dabrowski, J., P. Hauflend, and H. Egge.** 1980. Structural analysis of glycosphingolipids by high resolution $^1$H nuclear magnetic resonance spectroscopy. Biochemistry **19**:5652–5658.
3. **De Graaf, F. K., P. Klemm, and W. Gaastra.** 1980. Purification, characterization, and partial covalent structure of *Escherichia coli* adhesive antigen K99. Infect. Immun. **33**:877–883.
4. **De Graaf, F. K., and J. Roorda.** 1982. Production, purification, and characterization of the fimbrial adhesive antigen F41 isolated from calf enteropathogenic *Escherichia coli* strain B41M. Infect. Immun. **36**:751–758.
5. **Ernst, R. R.** 1966. Sensitivity enhancement in magnetic resonance, p. 1–135. *In* J. S. Waugh (ed.), Advances in magnetic resonance, vol. 2. Academic Press, London.
6. **Faris, A., M. Lindahl, and T. Wadström.** 1980. $GM_2$-like glycoconjugate as possible erythrocyte receptor for the CFA/I and K99 haemagglutinins of enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. **7**:265–269.
7. **Firon, N., I. Ofek, and N. Sharon.** 1982. Interaction of mannose-containing oligosaccharides with the fimbrial lectin of *Escherichia coli*. Biochem. Biophys. Res. Commun. **105**:1426–1432.
8. **Fukado, M. N., and S. I. Hakamori.** 1982. Structures of branched blood group A-active glycosphingolipids in human erythrocytes and polymorphism of A- and H-glycolipids in $A_1$ and $A_2$ subgroups. J. Biol. Chem. **257**:446–455.
9. **Gaastra, W., and F. K. de Graaf.** 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46**:129–161.
10. **Gasa, S., A. Makita, and Y. Kinoshita.** 1983. Further study of the chemical structure of the equine erythrocyte hematoside containing O-acetyl ester. J. Biol. Chem. **258**:876–881.
11. **Guinée, P. A. M., W. H. Jansen, and C. M. Agterberg.** 1976. Detection of the K99 antigen by means of agglutination and immunoelectrophoresis in *Escherichia coli* isolates from calves and its correlation with enterotoxigenicity. Infect. Immun. **13**:1369–1377.
12. **Hakomori, S.** 1964. Rapid permethylation of glycolipids and polysaccharides, catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55**:205–208.
13. **Jansson, P. E., L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren.** 1976. A practical guide to the methylation analysis of carbohydrates. Chem. Commun. Univ. Stockholm, vol. **8**.
14. **Källenius, G., R. Möllby, S. B. Svenson, J. Winberg, A. Lundblad, S. Svensson, and B. Cedergren.** 1980. The $p^k$ antigen as receptor for the hemagglutination of pyelonephritic *Escherichia coli*. FEMS Microbiol. Lett. **7**:297–302.
15. **Kamerling, J. P., L. Dorland, H. van Halbeek, J. F. G. Vliegenthart, M. Messer, and R. Schauer.** 1982. Structural studies on 4-O-acetyl-N-acetylneuroaminyl-α(2→3)-lactose, the main oligosaccharide in echidna milk. Carbohydr. Res. **100**:331–340.
16. **Kamerling, J. P., G. J. Gerwig, J. F. G. Vliegenthart, and J. R. Clamp.** 1975. Characterization by gas-liquid chromatography-mass spectrometry and proton-magnetic-resonance spectroscopy of pertrimethylsilyl methyl glycosides obtained in the methanolysis of glycoproteins and glycopeptides. Biochem. J. **151**:491–495.
17. **Kamerling, J. P., and J. F. G. Vliegenthart.** 1982. Gas-liquid chromatography and mass spectrometry of sialic acids. Cell Biol. Monogr. **10**:95–125.
18. **Karlsson, K. A., I. Pascher, W. Pimlott, and B. E. Samuelsson.** 1974. Use of mass spectrometry for the carbohydrate composition and sequene analysis of glycosphingolipids. Biomed. Mass Spectrom. **1**:49–56.
19. **Karlsson, K. A., B. E. Samuelsson, and G. O. Steen.** 1973. The sphingolipid composition of bovine kidney cortex, medulla and papillo. Biochim. Biophys. Acta **316**:317–335.
20. **Koerner, T. A. W., J. H. Prestegard, P. C. Demou, and R. K. Yu.** 1983. High-resolution proton NMR studies of gangliosides. I. Use of homonuclear two-dimensional spin-echo J-correlated spectroscopy for determination of residu composition and anomeric configuration. Biochemistry **22**:2676–2687.
21. **Leffler, H., and C. Svanborg-Edén.** 1981. Glycolipid receptors for uropathogenic *Escherichia coli* on human erythrocytes and uroepithelial cells. Infect. Immun. **34**:920–929.
22. **Ohashi, M., and T. Yamakawa.** 1977. Isolation and characterization of glycosphingolipids in pig adipose tissue. J. Biochem. (Tokyo) **81**:1675–1690.
23. **Parkkinen, J., J. Finne, M. Achtman, V. Väisänen, and T. K. Korhonen.** 1983. *Escherichia coli* strains binding neuraminyl α 2-3 galactosides. Biochem. Biophys. Res. Commun. **111**:456–461.
24. **Runnels, P. L., H. W. Moon, and R. A. Schneider.** 1980. Development of resistance with host age to adhesion of $K99^+$ *Escherichia coli* to isolated intestinal epithelial cells. Infect. Immun. **28**:298–300.
25. **Sellwood, R., R. A. Gibbons, G. W. Jones, and J. M. Rutter.** 1975. Adhesion of enteropathogenic *Escherichia coli* to pig intestinal brush borders: the existance of two pig phenotypes. J. Med. Microbiol. **8**:405–411.
26. **Stahl, E.** 1976. Dünnschichtchromatographie. Springer Verlag, Berlin.
27. **Svanborg Edén, C., B. Eriksson, and L. Å. Hanson.** 1977. Adhesion of *Escherichia coli* to human uroepithelial cells in vitro. Infect. Immun. **18**:767–774.
28. **van Embden, J. D. A., F. K. de Graaf, L. M. Schouls, and J. S. Teppema.** 1980. Cloning and expression of a deoxyribonucleic acid fragment that encodes for the adhesive antigen K99. Infect. Immun. **29**:1125–1133.
29. **Yamakawa, T., and S. Suzuki.** 1951. The chemistry of the lipides of posthemolytic residue or stroma of erythrocytes. I. The ether-insoluble lipides of lyophilized horse blood stroma. J. Biochem. (Tokyo) **38**:199–212.