Enhanced Binding of Enterotoxigenic *Escherichia coli* K99 to Amide Derivatives of the Receptor Ganglioside NeuGc-GM3†

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ABSTRACT: A natural receptor in pig small intestine [Teneberg, S., Willemsen, P., de Graaf, F. K., & Karlsson, K.-A. (1990) *FEBS Lett. 263*, 10−14] for the enterotoxigenic bacteria *Escherichia coli* K99 is the ganglioside NeuGc-GM3 (NeuGcα3Galβ4Glcβ Cer) [e.g., H. Smit, W. Gaastra, J. P. Kamerling, J. F. G. Vliegenthart, & F. K. de Graaf (1984) *Infect. Immun. 46*, 578−584]. Chemical modifications of the carboxyl group of this ganglioside were performed, giving five different amides, the methyl ester, and the primary alcohol. The products were purified, and their structures were investigated by negative FAB mass spectrometry. Binding of *E. coli* K99 was tested by incubating ^{35}S-labeled bacteria with derivatized compounds separated on thin-layer chromatograms. Modification of the carboxyl group to a primary amide strengthened the binding at least 5-fold, as estimated from autoradiography of dilutions on thin-layer plates. Some strengthening of the binding was also obtained with the methylamide as well as with the carboxyl group reduced to the alcohol. The ethylamide bound equally well as the underivatized NeuGc-GM3. Amide substituents as large as propyl amide and benzyl amide were still recognized by the bacteria, although they bound weaker. The methyl ester was not stable in the chromatogram-binding assay with silica gel and water present, and it reverted to the acid.

Enterotoxigenic *Escherichia coli* isolated from some domestic animals are often found to carry K99 fimbria either alone or together with other types of fimbria [reviewed by Jacobs et al. (1986)]. The bacteria cause severe, sometimes lethal diarrhea in piglets, lambs, and calves (Gaastra & de Graaf, 1982). Even if not expressed, the K99-carrying plasmid seems to be present in most enterotoxigenic *E. coli* (Isaacson & Start, 1992) and is thought to be of great importance for bacterial adhesion to target tissue (Mouricout & Julien, 1987).

Several natural ganglioside receptors for *E. coli* K99 have been isolated: NeuGc-GM3[1] (Smit et al., 1984; Kyogashima et al., 1989; Teneberg et al., 1990; Yuyama et al., 1993), NeuGc-nLc$_4$ (Kyogashima et al., 1989), NeuGc-GM2 (Teneberg et al., 1994), and NeuGcNeuGc-GD1a (Teneberg et al., 1994). Mucins from the intestine of susceptible animals are also recognized as receptors by the bacteria (Mouricout & Julien, 1987). Several factors seem to contribute to the *E. coli* K99 susceptibility of piglets. By analyses of the glycolipids present in target organs for the *E. coli* K99 it has been shown that piglets carry the receptor NeuGc-GM3, while older pigs mainly carry the inactive NeuAc-GM3 (Teneberg et al., 1990). NeuGc-GM3 with highly hydroxylated ceramide was found to be present predominantly in the intestine of young pigs and to be bound most strongly by *E. coli* K99 (Yuyama et al., 1993). Also, the gangliosides of pig strains that are more resistant to this enterotoxigenic bacterium were found to have relatively less NeuGc-GM3 and other binding gangliosides compared to susceptible strains (Seignole et al., 1991). Binding gangliosides in calf intestine were recently analyzed (Teneberg et al., 1994). In addition, it has been demonstrated that *E. coli* K99 binds to epithelial cells, *in vitro*, of newborn pigs, calves, and mice (Runnels et al., 1980), and some mice strains have in clinical experimental situations been found to be sensitive to enterotoxigenic *E. coli* K99 (Duchet-Suchaux et al., 1990).

For most *E. coli* K99 strains tested, NeuGc-GM3 was the most effective glycolipid in inhibiting hemagglutination of equine erythrocytes (Ono et al., 1989). This ganglioside was shown to be at least 10 times as effective as NeuAc-GM3, 4-*O*-Ac-NeuGc-GM3, and NeuGc-nLc$_6$Cer. Kyogashima et al. (1989), however, found that NeuGc-nLc$_4$Cer bound the bacteria about 10 times better than NeuGc-GM3 when tested by the TLC overlay method. In addition, *E. coli* K99 bound to NeuGc-GM2 and NeuAc-nLc$_4$Cer on TLC plates, but not to NeuAc-GM3 or NeuGc-GM1 (Teneberg et al., 1990, 1994). The gangliosides GQ1b and GT1b, isolated from piglets susceptible to *E. coli* K99 infection, also bound the bacteria on TLC overlay analysis (Seignole et al., 1991).

In an attempt to reveal the detailed interaction between the receptor ganglioside NeuGc-GM3 and the bacterial adhesin, we have made a series of chemically modified NeuGc-GM3. These all have in common that the negative charge of the carboxyl group of the sialic acid is neutralized, via either a reduction to a primary alcohol or a transesterification with different amines. The binding of *E. coli* K99

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[1] Abbreviations: FAB, fast atom bombardment; EI, electron impact; MS, mass spectrometry; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Cer, ceramide; Neu, neuraminic acid; NeuGc, *N*-glycolylneuraminic acid; NeuAc, *N*-acetylneuraminic acid; NeuGc-GM3, NeuGcα3Galβ4Glcβ Cer; GM2, GalNAcβ4(NeuAcα3)-Galβ4Glcβ Cer; GM1, Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ Cer; nLc$_4$, Galβ4GlcNAcβ3Galβ4Glcβ Cer; NeuGc-nLc$_6$Cer, NeuGcα3Galβ4-GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ Cer; NeuGcNeuGc-GD1a, NeuGcα3Galβ3GalNAcβ4(NeuGcα3)Galβ4Glcβ Cer; GT1b, Neuα3-Galβ3GalNAcβ4(Neuα8Neuα3)Galβ4Glcβ Cer; GQ1b, Neuα8Neuα3-Galβ3GalNAcβ4(Neuα8Neuα3)Galβ4Glcβ Cer. Ganglioside abbreviations like GM1, GM2, etc. are according to Svennerholm (1980).

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to the primary amide was found to be increased compared to the binding of the unmodified receptor.

MATERIALS AND METHODS

NeuGc-GM3 was isolated from horse erythrocytes, and NeuAc-GM1 from human brain, according to Karlsson (1987). The isolated ganglioside was characterized in native form by negative FABMS and in permethylated and permethylated/reduced/methylated form by EIMS (Karlsson, 1974).

The ganglioside (0.5 mg) was first transformed to the methyl ester by reaction with methyl iodide (100 μL) in dimethyl sulfoxide (0.5 mL) as described by Handa and Nakamura (1984). The reaction was allowed to proceed at room temperature for 1 h, with repeated ultrasonic treatments for 5 min. Prolonged reaction time or increased temperature resulted in darkening of the reaction mixture. After lyophilization, the products were dissolved in 0.5 mL of methanol, and for production of the amide 0.2 mL of NH_3 (30% in H_2O) was added. To obtain the methyl, ethyl, propyl, and benzyl amide, respectively, the following additions were made: 0.5 mL of methylamine (40% in H_2O) (Nakamura & Handa, 1986), 1 mL of ethylamine (70% in H_2O), 75 μL of propylamine (99%), and 75 μL of benzylamine (99%) (all from Aldrich, Steinheim, Germany). The reaction conditions used were 12 h at room temperature in all cases except for the propyl and benzyl amides, where 1 h was found to be sufficient. No systematic investigation of the optimal amine proportions, reaction times, or temperatures was made. In the next step the reaction mixtures were carefully dried, either by evaporation with a stream of N_2(g) or by lyophilization. Very small amounts of unreacted acid remained and were removed together with salts by Folch separation in chloroform/methanol/water, 8:4:3 (all mixtures expressed as v:v unless otherwise stated). The amides were collected in the lower (hydrophobic) phase, and this phase was washed twice with 2 mL of Folch upper phase. Alternatively, the amides were purified by ion-exchange chromatography on a DEAE-Sepharose (Pharmacia, Sweden) column, 10 × 100 mm, equilibrated and eluted with chloroform/methanol, 2:1.

Amide and benzyl amide derivatives of the sialic acid carboxyl group of GM1 were prepared in the same way as the NeuGc-GM3 derivatives.

To obtain the primary alcohol of NeuGc-GM3, 0.5 mg of NeuGc-GM3 methyl ester, synthesized as above, was dissolved in 0.5 mL of methanol, and 5 mg of $NaBH_4$ was added (Handa & Nakamura, 1984). After 1 h at room temperature, 10 mL of water was added. Addition of 12 mL of methanol and 25 mL of chloroform was done to obtain a two-phase system, and the mixture was dialyzed for 4 days.

For monitoring of purifications, glass-backed TLC plates were used, and for separation and bacterial binding tests alumina-backed plates were used (HPTLC, 0.2 mm nanoplates; Merck, Germany). The TLC separations in Figures 1, 4, 5, and 6 were made with chloroform/methanol/water, 60:35:8. Dimethyl sulfoxide was purchased from Merck, and CH_3I was from Fluka, Germany. Chemical detection of glycolipids was done by spraying with either anisaldehyde (Waldi, 1962) or resorcinol (Svennerholm, 1963, and references therein). Quantitation of glycolipids was done by spectrophotometric determination of sphingosine in complex with methyl orange as described by Lauter and Trams (1962).

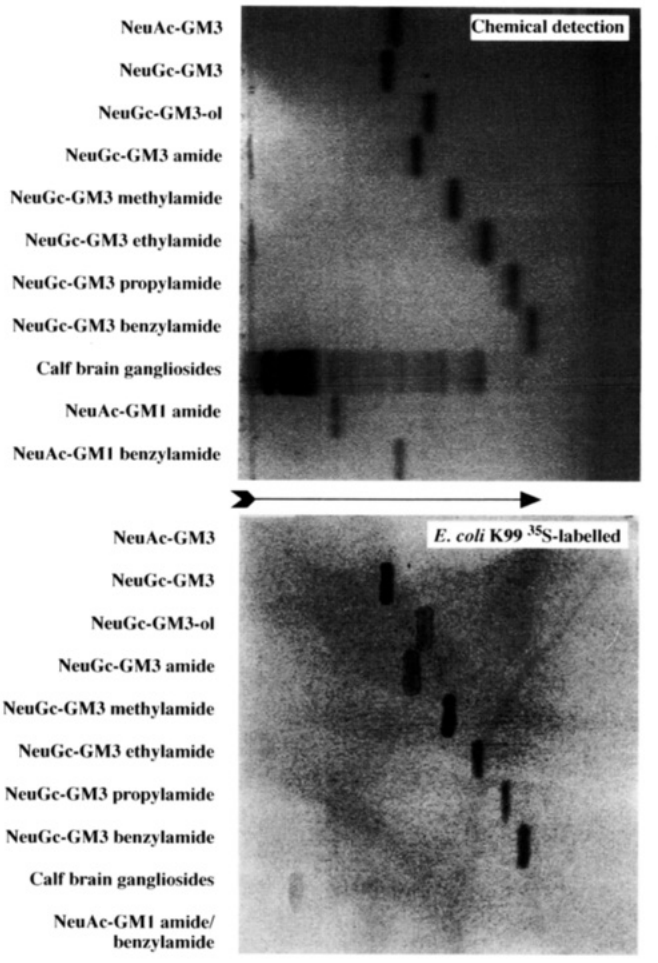


FIGURE 1: TLC separation of native and chemically modified gangliosides. In the top chromatogram, the glycolipids were detected by anisaldehyde, and in the bottom one, by autoradiography after incubation with ^{35}S-labeled *E. coli* K99. The amount of each glycolipid applied per lane was 3−5 nmol.

For characterization of starting material and reaction products negative FABMS and EIMS analyses were done with a VG ZAB 2F/HF double-focusing mass spectrometer equipped with a Xe atom gun operated at 8−9 kV. The FAB probe tip used had a small sample cup with a diameter of 3 mm and a depth of 0.5 mm. By this shape the matrix, triethanolamine, was kept in place during the entire MS operation. Better control of the temperature of the sample and easier handling were other features of this new design. The mass assignments can deviate 1 mass unit from theoretical values for ions of low abundance. Mass spectra were processed with a Mach-3 work station (Kratos, U.K.).

Proton-NMR spectra were recorded with a Varian VXR 300 spectrometer (Varian, Palo Alto, Ca., USA) operating at 300 MHz and 7.05 T. The solvent used was $CDCl_3$.

Bacteria, a gift from Dr. Willemsen, Vrie University, Amsterdam, were cultivated in MINCA medium (Guinée et al., 1977) and labeled with [^{35}S]methionine as described by Teneberg et al. (1990). The bacterial overlay technique was described by Karlsson and Strömberg (1987). The TLC plate on which the glycolipids were chromatographed was dipped into a solution with 0.3% poly(isobutyl methacrylate) (Plexigum P28, Röhm GmbH, Darmstadt, Germany) in diethyl ether/hexane, 3:1, dried, soaked in 2% BSA in PBS (8 mM phosphate buffer, pH 7.3, 0.14 M NaCl, and 4 mM KCl) for

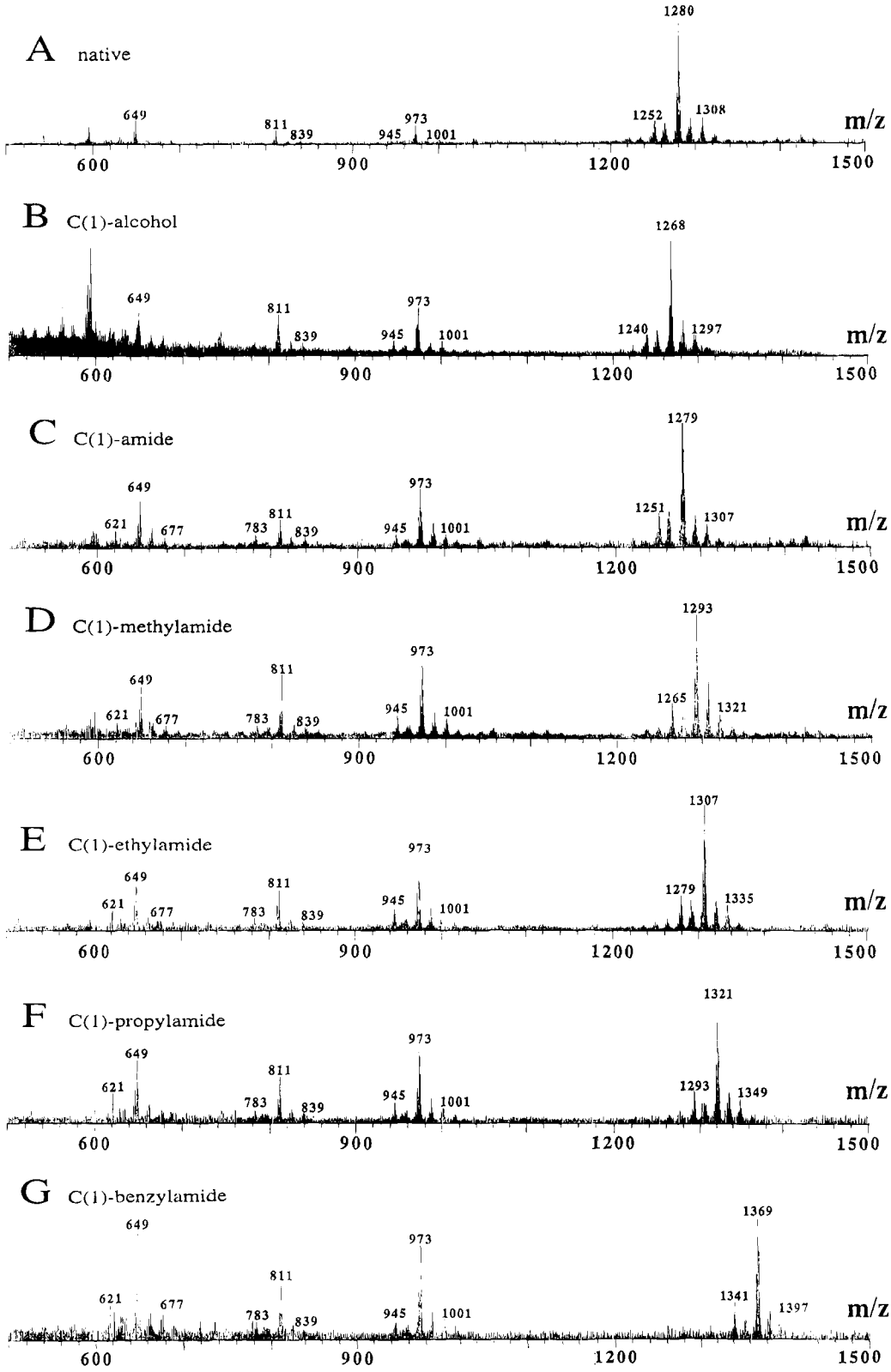
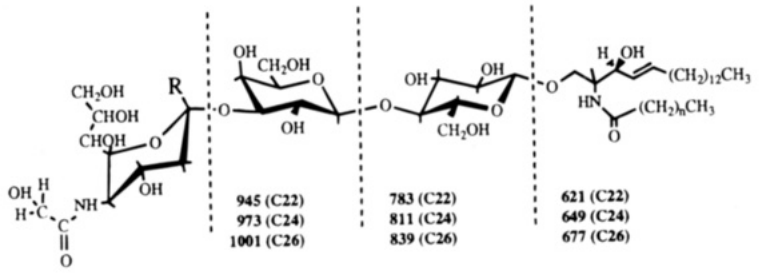


FIGURE 2: Negative FAB mass spectra of chemically modified NeuGc-GM3. Spectra of the following compounds are shown: A, underivatized NeuGc-GM3; B, primary alcohol, obtained after reduction with $NaBD_4$; C, primary amide of NeuGc-GM3; D, methyl amide of NeuGc-GM3; E, ethyl amide of NeuGc-GM3; F, propyl amide of NeuGc-GM3; and G, benzyl amide of NeuGc-GM3.

2 h, incubated with bacterial solution in PBS for another 2 h, washed 5 times with PBS, and dried.

To test the stability of the ganglioside amides under the conditions used for overlay with bacteria on TLC plates, the following tests were done. Purified amide derivatives of GM3 were incubated for 4 h in two buffer systems, in PBS as described above and in 2.4 mM CH_3COONa, pH 4.8, 0.14 M NaCl, and 5 mM KCl, in the presence of a few milligrams of silica gel (scraped from alumina-backed TLC plates). The tubes were vigorously shaken for 1 min each hour. The silica gel was filtered off, and the samples were redissolved in chloroform/methanol, 2:1, and analyzed by TLC. The stability of the GM3 methyl ester was tested by two-dimensional TLC with exposure to a humid atmosphere at


945 (C22)
973 (C24)
1001 (C26)
783 (C22)
811 (C24)
839 (C26)
621 (C22)
649 (C24)
677 (C26)

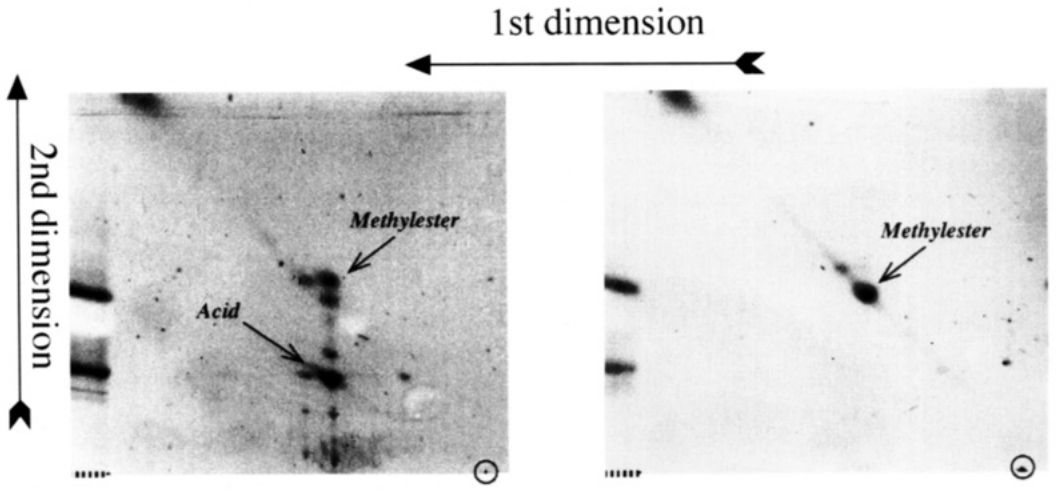


FIGURE 5: Two-dimensional TLC of the methyl ester of NeuGc-GM3 with different treatments of the TLC plate between the two developments. The plate on the left was kept for 4 h in a humid atmosphere at 50 °C, and the plate on the right was kept in a desiccator at 25 °C. (○) Application point for two-dimensional TLC; (- - -) application line for one-dimensional TLC of references of native NeuGc-GM3 and its methyl ester. The glycolipids were visualized with resorcinol staining.

new signal appeared at 2.6 ppm (not shown).

The stability of the amides was tested by incubation with silica gel in buffer at pH 7.4 and 4.8 for 4 h. After removal of the silica gel the samples were chromatographed on TLC plates. Neither chemical detection with anisaldehyde (not shown) nor incubations of the treated amides with labeled *E. coli* K99 showed binding bands other than those of the expected amide derivatives (Figure 4). The methyl ester, on the other hand, was not stable in a water milieu on TLC plates. Two-dimensional TLC analysis (Figure 5) was performed where the plate was incubated between the developments in a humid atmosphere for 4 h at 50 °C (Figure 5, left). A substantial formation of the acid occurred, while storage of the plate in a desiccator for 4 h left the methyl ester intact (Figure 5, right). Treatment of the benzyl amide derivative in exactly the same way gave no degradation products (not shown). Conversion of the methyl ester into

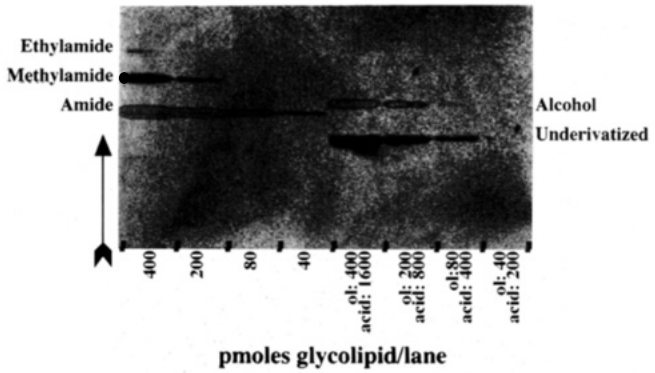


FIGURE 6: *E. coli* K99 binding to serial dilutions of native and derivatized NeuGc-GM3 after separation by TLC. In the left four lanes ethyl amide, methyl amide, and primary amide were applied together at 400, 200, 80, and 40 pmol/lane, and in the right four lanes the alcohol and the native compound were applied together at 400 and 1600, 200 and 800, 80 and 400, and 40 and 200 pmol/

derivatives, both the alcohol and the amides, had a slightly increased tendency to loose the sialic acid compared to the native compound. The amide bond, however, was apparently stable under these circumstances. The proton-NMR spectrum of the primary amide did not differ from that of the acid. The amide protons are exchanging with traces of water present. In the spectrum of the methyl amide, however, a new signal was found at 2.6 ppm. Nakamura and Handa (1986) reported a signal at 2.68 ppm for the methyl amide derivative of GM1.

All derivatives, both the alcohol and the amides, gave a blue color with resorcinol when applied in normal amounts on the TLC plate (0.5−2 nmol). Handa and Nakamura (1984), however, reported that the alcohol gave pink-yellow staining with resorcinol.

It is of importance that the stability of the derivatives be tested. Also, small amounts of unreacted or degraded material may affect the binding results. The methyl esters of gangliosides have been used in some biological studies (Handa & Nakamura, 1984; Li et al., 1984; Nakamura & Handa, 1986; Hanisch et al., 1993). Our data, however, clearly showed that this compound was not stable in the presence of silica gel and water. Therefore, one should be careful in using this derivative for biological testing. Whether this derivative is stable in biological fluids is not known, although the possible lability of the methyl ester of GM1 was discussed (Nakamura & Handa, 1986).

Binding of *E. coli* K99 to chemically modified gangliosides was used here for the first time. Binding to a naturally occurring receptor analogue, 4-*O*-Ac-NeuGc-GM3, has been tested (Ono et al., 1989), and it showed a 500-fold decrease of binding when measured as inhibition of hemagglutination.

The binding specificity of *E. coli* K99 has been studied recently by Teneberg et al. (1994). The same bacterial isolate as used by us was analyzed for binding to 15 different naturally occurring gangliosides with structural similarity to NeuGc-GM3, and by use of molecular modeling a binding epitope was proposed. The general conclusion was that a glycolyl group is required. However, the penultimate Gal of NeuAcα3Galβ4GlcNAcβ3Galβ4GlcCer can apparently substitute for a glycolyl group, which explains the relatively strong binding to this ganglioside.

Chemical modification of amino acids of the isolated adhesin in the presence or absence of NeuGc-GM3 indicated that two of the eight lysines present, Lys 132 and Lys 133, were probably involved in the binding (Jacobs et al., 1986). These lysines are located in a stretch of six amino acids with great homology to the GM1-binding sequence of heat-labile toxin of *E. coli* and of the binding subunit of cholera toxin (Jacobs et al., 1986). The importance of Lys 132 was confirmed by site-directed mutagenesis (Jacobs et al., 1987), where exchange to Thr abolished the GM3 binding completely. Also, Arg 136 was essential. The authors suggested that there is a charge interaction between the sialic acid carboxyl group and lysine residues of the protein. Since our data showed that the amide derivative of GM3 bound better than the native compound, it is more probable that hydrogen bonding is important. The alcohol and the methyl amide would be less suited for hydrogen bonding than the acid or the primary amide, but they still bind slightly better than the native receptor.

Recently, glycoconjugates of bovine blood plasma, when given in drinking water, were shown to cure newborn calves infected with lethal doses of *E. coli* K99 (Mouricout et al., 1990). Probably, as suggested by the authors, di-, tri-, and tetraantennary sequences terminating in Neu(Ac/Gc)α3-Galβ4GlcNAc- were responsible for the inhibition of bacterial adhesion in the small intestine. This is the first report of an antiadhesive treatment in a clinically relevant situation. Detailed knowledge of the adhesin−saccharide interaction may form a rational basis for synthetic saccharide analogues, which are more effective and may be easier to prepare than the natural compounds. Toward this end, our result of at least 5-fold better binding of the primary amide of GM3 could be of importance.

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