Glycoconjugate J (1987) 4: 51-58

Carbohydrate Receptor Specificity of K99 Fimbriae of Enterotoxigenic *Escherichia coli*

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Received November 14, 1986.

Key words: carbohydrate receptor, E.coli K99, sialic acid

K99 Fimbriae from enterotoxigenic *Escherichia coli* (ETEC) were found to bind specifically to sialic acid, as measured in a haemagglutination inhibition assay using the intact bacteria and human erythrocytes. The affinity for *N*-glycolylneuraminic acid was about twice that of *N*-acetylneuraminic acid (NeuAc), and other monosaccharides were found to be at least ten-fold less effective as inhibitors. The specificity was found to depend on electrostatic interaction where the carboxyl group and its orientation plays an important role. 2-α-Benzyl-NeuAc was a better inhibitor than 2-α-methyl-NeuAc suggesting a hydrophobic patch near the binding site on the protein. Axially oriented hydroxyl groups as in 4-epi-NeuAc and 3-hydroxy-NeuAc seemed to participate in binding since these derivatives were better inhibitors than *N*-acetylneuraminic acid. K99 was found to have a higher affinity for 4-*O*-acetyl-NeuAc and lower affinity for *N*-acetylneuraminic acid with *O*-substituents at C7-C9 as compared to *N*-acetylneuraminic acid. Hence, the degree of *O*-acetylation of sialic acid in the mucosa of the small intestine may influence colonization and determine susceptibility to infection.

K99 Fimbriae are often produced by enterotoxigenic *Escherichia coli* (ETEC) isolated from piglets, calves and lambs suffering from diarrhoea [1, 2]. In addition, other fimbriae, e.g. type 1 and F41 fimbriae, are often produced by these strains. The K99 fimbria is a basic, homopolymeric protein composed of subunits with a molecular weight of 18 400 [3]. Morphologically, K99 is 4.8 nm in diameter and protrudes about 0.5 μm from the cell surface, as demonstrated by electron microscopy. K99-Fimbriated bacteria agglutinate erythrocytes of equine, human, porcine, bovine and ovine origin in the presence of D-mannose. The affinity of K99 for sialic acid has been reported [4] and the haemagglutination has been found to be weaker after sialidase treatment of erythrocytes [5].

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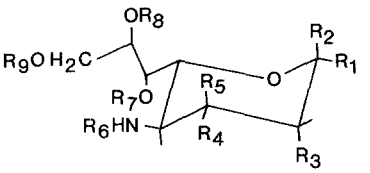
Table 1. Structures of sialic acid derivatives.



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Substance	1	2	3	4	5	6	7	8	9
NeuAc	COOH	OH	H	OH	H	Ac	OH	OH	OH
2-α-Benzyl-nonulosamine	OBz	CH_2OH	H	OH	H	Ac	OH	OH	OH
2-α-Methyl-NeuAc	OMe	COOH	H	OH	H	Ac	OH	OH	OH
2-β-Methyl-NeuAc	COOH	OMe	H	OH	H	Ac	OH	OH	OH
2-α-Benzyl-NeuAc	OBz	COOH	H	OH	H	Ac	OH	OH	OH
2-α-Benzyl-NeuAc-methylester	OBz	$COOCH_3$	H	OH	H	Ac	OH	OH	OH
2-α-Benzyl-NeuAc-amide	OBz	$CONH_2$	H	OH	H	Ac	OH	OH	OH
3-Hydroxy-NeuAc	COOH	OH	OH	OH	H	Ac	OH	OH	OH
4-*O*-Acetyl-NeuAc	COOH	OH	H	OAc	H	Ac	OH	OH	OH
4-epi-NeuAc	COOH	OH	H	H	OH	Ac	OH	OH	OH
NeuGc	COOH	OH	H	OH	H	$COCH_2OH$	OH	OH	OH
2-α-Benzyl-5-nor-acetyl-NeuAc	OBz	COOH	H	OH	H	H	OH	OH	OH
4,7-Di-*O*-acetyl-NeuAc	COOH	OH	H	OH	H	Ac	Ac	OH	OH
2-α-Benzyl-8,9-isopropylidene-NeuAc	OBz	COOH	H	OH	H	Ac	OH	isopropyl	
2-α-Benzyl-9-*O*-acetyl-NeuAc	OBz	COOH	H	OH	H	Ac	OH	OH	Ac
9-*O*-Acetyl-NeuAc	COOH	OH	H	OH	H	Ac	OH	OH	Ac

Ac = $COCH_3$
Bz = $CH_2C_6H_5$
Me = CH_3

By binding to receptors in the small intestine of the young animal, K99 fimbriae enable ETEC strains to colonize. Newborn piglets are most sensitive to infection by K99-producing *E. coli*, but gradually become resistant up to about two weeks of age, when they become completely resistant [6]. Adhesion of the pathogen to epithelial cells from the small intestine of pigs and calves has also been shown to depend on the age of the animal in a similar way [7].

Materials and Methods

Chemicals

Casamino acids and Bacto agar, were products of Difco Laboratories (Detroit, MI, USA). Octyl-Sepharose was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). *N*-Acetylneuraminic acid, *N*-glycolylneuraminic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-fucose, and methyl α-D-mannoside were obtained from Sigma

Chemical Co. (St. Louis, MO, USA). D-Glucose was obtained from BDH Chemicals Ltd (Poole, England). D-Galactose was purchased from Fluka AG (Buchs, Switzerland). Sialic acid derivatives (Table 1) were synthesized and the sialyllactoses were purified at the Department of Biochemistry in Heidelberg, West Germany. Detailed procedures will be published elsewhere.

Bacterial Cells

Strain B117 (08:K85ab, K99:H$^-$), an enterotoxigenic *E. coli* of bovine origin, was kindly supplied by Dr J.A. Morris, Central Veterinary Laboratory, Weybridge, England. Cultivation at 37°C was performed on Minca-agar containing: KH_2PO_4, 1.36 g; $Na_2HPO_4.2H_2O$, 10.1 g; D-glucose, 1 g; trace salts solution, 1 ml; Casamino acids, 1 g; Bacto agar, 12 g; and distilled water, 1000 ml; the pH was adjusted to 7.5. The trace salts solution contained, per liter: $MgSO_4.7H_2O$, 10 g; $MnCl_2.4H_2O$, 1 g; $FeCl_3.6H_2O$, 0.135 g; and $CaCl_2.H_2O$, 0.4 g [8].

Fimbriation of Bacterial Cells

The amount of fimbriated bacterial cells was assessed by hydrophobic interaction chromatography. Pasteur pipettes were plugged with glass wool and packed with Octyl-Sepharose to a bed volume of about 0.5 ml. The column was equilibrated with 0.01 M sodium phosphate buffer, pH 6.8, containing 1 M ammonium sulphate. A bacterial suspension of 10^{10} cells/ml (100 μl) was applied, followed by 2 ml buffer, as mentioned above [9]. Visual comparison of eluate with dilutions of bacterial suspension was made to evaluate the amount of eluted bacteria. The percentage of eluted bacteria was regarded as the non-fimbriated fraction of the cell population and the adsorbed bacteria as fimbriated. Bacterial suspensions containing more than 85% fimbriated cells were used in the haemagglutination inhibition experiment.

Erythrocytes

Human blood was collected with a heparinized syringe and washed three times in 10 vol of 0.11 M sodium phosphate buffer, pH 7.4.

Haemagglutination Inhibition Test

Inhibitors were mixed with suspensions of human erythrocytes (5×10^6 cells/ml) in a 1:1 mixture of 10 mM sodium phosphate/0.15 M NaCl, pH 7.4 (PBS) and 0.3 M sucrose containing 0.1 M methyl α-D-mannoside in a cell aggregometer (Payton 300 A, Payton Assoc. Inc., Buffalo, NY, USA) at 400 rpm and 20°C. The transmitted light was measured for 5-10 min to assure that inhibitors did not cause any change in transmitted light. Bacterial cells (4×10^7 fimbriated cells/ml) were added and the slope, i.e. the increase of transmitted light per minute at 609 nm, was measured. An increase in transmittance is a measure of the decreasing number of particles (i.e. erythrocytes or clumps of erythrocytes) scattering light, as the haemagglutination reaction progresses.

The increase in transmittance is constant for a few minutes, after a lag phase of 1-2 min [4]. The slope during the constant phase, obtained after addition of inhibitors at dif-

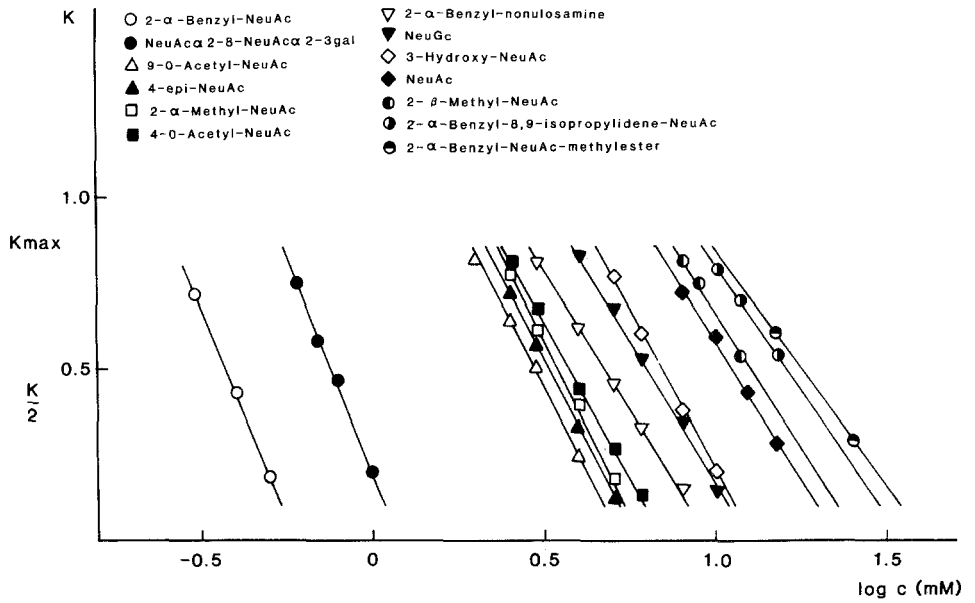

Figure 1. Slopes (K) of haemagglutination reactions in the presence of inhibitors plotted against log concentrations of inhibitors. K_{max} = 0.860 and represents haemagglutination without inhibitor. K/2 = 0.430 and represents 50% inhibition.

ferent concentrations, was plotted against the log concentration of inhibitor (slope/log c), giving linear plots for each inhibitor (Fig. 1). The concentration of each inhibitor reducing, by 50%, the increase in transmittance per minute was determined [10]. The mean deviation within an experiment with every single inhibitor was calculated as ± 5%. The pH was always measured after each test to assure that the inhibition was not due to a decreased pH-value.

Pretreatment of Sialic Acid and its Acidic Derivatives

Sialic acids were used throughout the study as ammonium salts in order to keep the pH at 7.4. The sialic acid ranging from 2-5 mg was dissolved in 0.2 ml H_2O and an equimolar amount of 0.1 M NH_4OH was added. The mixtures were freeze-dried and the resulting ammonium salts were dissolved in PBS.

Results

Haemagglutination Inhibition

Sialic acids were found to be the most efficient inhibitors of haemagglutination with K99-fimbriated cells. D-Galactose, L-fucose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-

Table 2. Inhibition of K99 haemagglutination with monosaccharides. Haemagglutination inhibition was performed in an aggregometer as described in the Materials and Methods section.

Sugar	50% Inhibition (M)
GlcNAc	0.30
GalNAc	0.23
Fuc	0.16
Gal	0.12
NeuAc	0.012
NeuGc	0.0068

Table 3. Inhibition of K99 haemagglutination with sialic acid derivatives. Haemagglutination inhibition was performed in an aggregometer as described in the Materials and Methods section.

Derivative	50% Inhibition (mM)
NeuAc	12
2-α-Benzyl-nonulosamine	5.2
2-α-Methyl-NeuAc	3.7
2-β-Methyl-NeuAc	14
2-α-Benzyl-NeuAc	0.4
2-α-Benzyl-NeuAc-methylester	20
2-α-Benzyl-NeuAc-amide	1.1×10^2
3-Hydroxy-NeuAc	6.9
4-*O*-Acetyl-NeuAc	4.1
4-epi-NeuAc	3.5
NeuGc	6.8
2-α-Benzyl-5-noracetyl-NeuAc	6.0[a]
4,7-di-*O*-Acetyl-NeuAc	2.5[a]
2-α-Benzyl-8,9-isopropylidene-NeuAc	18
2-α-Benzyl-9-*O*-acetyl-NeuAc	3.2
9-*O*-Acetyl-NeuAc	3.0[a]
NeuAcα2-3Galβ1-4Glc	3.5
NeuAcα2-6Galβ1-4Glc	3.1
NeuAcα2-8NeuAcα2-3Galβ1-4Glc	0.8

[a] No inhibition at this concentration, haemagglutination reaction appeared as the control.

glucosamine were 10, 15, 20 and 25 times less active inhibitors (Table 2). Increased inhibition was obtained when *N*-acetylneuraminic acid was 2-α-methylated, 2-α-benzylated or 3-hydroxylated; also when the hydroxyl group at C-4 was acetylated or changed to an axial position, and when C-5 was glycolylated instead of acetylated (Table 3 and Fig. 1). Decreased inhibition was detected when the carboxyl group was esterified or reduced to an alcohol. The inhibiting activity was decreased when the C-5 position was deacetylated, C-7 or C-9 acetylated, or when the hydroxyl groups at C-8 and C-9 were not available as in the 8,9-isopropylidene-derivative. α(2-8)-Disialyllactose was found to be a better inhibitor than α(2-3)- and α(2-6)-sialyllactose (Table 3).

Discussion

Purified human glycophorin or Fab fragments of rabbit IgG directed against human N-antigen have been shown to inhibit K99 haemagglutination [4]. A haematoside from horse erythrocytes has also been shown to be an inhibitor of K99 haemagglutination [11]. This is consistent with our results since sialic acid, which is present in the haematoside structure, is the specific binding site for K99 fimbriae.

The sialic acid specificity of K99 was found to be highly dependent on the presence of hydroxyl groups at C-7 to C-9, on an acetylated amine at C-5, and on an axially oriented carboxyl group at C-1. Loss of any of these groups resulted in at least a ten-fold decrease of inhibiting activity. However, it is possible that the introduction of acetyl groups at C-7 to C-9 leads to steric hindrance which accounts for the decrease of inhibiting activity. The introduction of an acetyl group at C-7, as in 4,7-diAc-NeuAc, may also change the orientation of C-7 to C-9, and thus change the intramolecular position of the hydroxyl groups at C-8 and C-9, resulting in a lowered inhibiting potency. 2-β-Methyl-NeuAc, with the carboxyl group at the equatorial position, had a four times lower inhibitory effect than 2-α-methyl-NeuAc, which has its carboxyl group in an axial position (Table 3). In fact, the free *N*-acetylneuraminic acid also has the carboxyl group in the equatorial position which explains why 2-β-methyl-NeuAc and *N*-acetylneuraminic acid have comparable inhibitory effects. Thus, the position of the carboxyl group of *N*-acetylneuraminic acid is important for inhibitory activity.

An additional hydroxyl group in the axial position at C-3 of *N*-acetylneuraminic acid enhances the inhibiting activity to a similar degree as when the additional hydroxyl group is placed on the *N*-acetyl group at C-5, as in *N*-glycolylneuraminic acid. It is probable that this enhancement is due to introduction of additional hydrogen bonds.

The carboxyl group of sialic acid is necessary for an electrostatic interaction with the positively charged protein. It has been shown that a low ionic strength promotes the haemagglutination reaction [4], which implies that the driving force of this interaction is mainly electrostatic. However, the 2-α-benzylated *N*-acetylneuraminic acid has a ten-fold higher inhibiting activity than the 2-α-methyl-NeuAc derivative (Table 3), suggesting that there is a hydrophobic region near the binding site of *N*-acetylneuraminic acid. Mannose-specific type 1 fimbriae of *E. coli* have also been shown to possess a hydrophobic region close to the carbohydrate binding site. When the specific monosaccharide was linked to an aromatic group, inhibition of type 1 haemagglutination was enhanced thirty-fold [10].

E. coli type 1 and P-fimbriae have both been shown to bind di- or trisaccharides better than monosaccharides [10, 12]. Similar findings have also been reported for many plant lectins, e.g. *Sophora japonica* lectin [13]. K99 showed a similar tendency since α(2-3)- and α(2-6)-sialyllactose, purified from bovine colostrum, had almost equal inhibiting activity, but higher than the *N*-acetylneuraminic acid monosaccharide (Table 3). This can be explained by the fact that the carboxyl group in α-linked sialic acid has an axial position while free sialic acid has the carboxyl group in an equatorial position (Table 1). The tetrasaccharide, α(2-8)-disialyllactose, had an even higher inhibiting activity which may be due to the additional negative charge conferred by the second sialyl residue. We have previously concluded that the α(2-6) linkage of *N*-acetylneuraminic acid to an oligosaccharide was superior to the α(2-3) linkage for the inhibition of haemagglutina-

tion [4]. However, those results were obtained with sialoglycoproteins having a different number of sialic acid residues. The degree of *O*-acetylation was not determined and the importance of the penultimate sugar was speculated. Some of the results of the present study are contradictory to our earlier findings where *N*-acetyl-D-galactosamine and *N*-acetylneuraminic acid were shown to be equally good inhibitors [4]. In the previous study a bacterial strain was used which by immunoprecipitation was found to produce only K99 fimbriae. However, it is probable that F41 fimbriae, which are specific for *N*-acetyl-D-galactosamine, were also present [14].

At present K99 is the most extensively studied sialic acid-specific lectin regarding binding specificity. However, there are a number of sialic acid specific lectins from other sources than bacteria, whose binding specificity is known to some extent. Limulin has been studied for several years and has a rather well characterized binding specificity. Electrostatic interaction occurs between the lectin and the carboxyl group [15], which is similar to the specific binding of K99. The acetyl group of *N*-acetylneuraminic acid is crucial for specific binding of both Limulin [16] and K99.

Limulin has a higher affinity for 2-α-methyl neuraminic acid than for 2-β-methyl neuraminic acid [16] although the difference is not as prominent as that of K99. Limulin does not bind at all to 4-*O*-acetylated sialic acid [17] in contrast to K99 (Table 3). The major difference between Limulin and K99 is that Limulin does not interact with the hydroxyl groups at C-7 to C-9 of sialic acid (Table 3).

Another sialic acid specific lectin has been isolated from *Cancer antenarius* [18]. This lectin preferentially binds to *O*-acetylated *N*-acetylneuraminic acid. The affinity for 9-*O*-acetylated *N*-acetylneuraminic acid is somewhat higher than for 4-*O*-acetylneuraminic acid, in contrast to K99.

A lectin isolated from *Limax flavus* is probably the most specific of all known sialic acid specific lectins [19], however, the specificity for *O*-acetylated sialic acids has not been studied for this lectin.

The similarities in glycoconjugate affinity between bacterial sialidases and K99 consist of the low affinity for sialic acids lacking the carboxyl group [20] and for 7-, 8- or 9-*O*-acetylated sialic acids [21, 22]. Sialidases have no affinity for β-glycosides [23] and sialic acids lacking the acetyl-group at C-5 [24]. The major difference is that sialidases have significantly lowered affinity for 4-*O*-NeuAc while K99 has a significantly higher affinity for this derivative as compared to *N*-acetylneuraminic acid [21]. The significance of the diverse specificity of K99 towards *N*-acetylneuraminic acid with different types of *O*-acetylation remains to be elucidated. However, it is known that enterotoxigenic *E. coli* with K99 fimbriae infects and causes diarrhoea in piglets, calves and lambs from the day of birth up to a few weeks of age and that the organism adheres to epithelial cells from the small intestine of calves solely during this period [7]. Furthermore, it has been claimed that sialic acids in the mucosa of young animals are less *O*-acetylated compared to adults [25]. Thus the degree of *O*-acetylation might influence the susceptibility to infection by K99 fimbriated enterotoxigenic *E. coli*.

Acknowledgements

The skilful technical assistance of Ms. Laila Eriksson and Mrs. Ursula Rose is gratefully

acknowledged. This work was supported by a grant from the Swedish Medical Research Council (16X0473), and by Funds der Chemischen Industrie.

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