Glycoconjugate Journal (1992) **9**:21–26

Detection of the ganglioside *N*-glycolyl-neuraminyl-lactosyl-ceramide by biotinylated *Escherichia coli* K99 lectin

ABDELOUAHAB OUADIA, YANNIS KARAMANOS* and RAYMOND JULIEN

Institut de Biotechnologie, Université de Limoges, 123, avenue Albert Thomas, 87060 Limoges cedex, France

Received 15 July, 1991

K99 lectin from *Escherichia coli* was purified and biotinylated via its carboxyl groups using biocytin hydrazide and a water soluble carbodiimide. Biotinylation of two out of the nine carboxyl groups was sufficient to permit detection of the lectin by avidin and did not cause any loss of the haemagglutinating activity. It was demonstrated that the biotinylated K99 lectin retained other important properties of native K99 and that it will probably become a very sensitive detecting reagent. Indeed, it was able to bind to HeLa cells, as do intact bacteria carrying K99 fimbriae, and also to recognize *N*-glycolyl-neuraminyl-lactosyl-ceramide in an overlay binding assay.

Keywords: K99 lectin, NeuGc-GM3 detection.

Abbreviations: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PBS, phosphate buffered saline (0.9% NaCl containing 150 mM sodium phosphate, pH 7.2); LPS, lipopolysaccharide; BCHZ, biocytin hydrazide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle medium. For the gangliosides, trivial names and structures are given according to the recommendations in [43]. NeuAcα2-3Galβ1-4Glcβ1-1Cer (NeuAc-GM_3); NeuGcα2-3Galβ1-4Glcβ1-1Cer (NeuGc-GM_3); GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer (GM_2); NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer (GD3); Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer (GM_1); NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer (GD_{1a}); Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer (GD_{1b}); NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1.-4Glcβ1-1Cer (GT_{1b}). NeuGcα2-3Galβ1-4GlcNAcβ1-4Galβ1-4Glcβ1-1Cer (NeuGc-SPG).

The enterotoxigenic *Escherichia coli* (ETEC) strains isolated from calves, lambs and piglets frequently express the K99 fimbrial antigen [1, 2] often associated with other fimbriae, e.g., F41 [3], FY [4]. A glycolipid identified as *N*-glycolyl-GM3 (NeuGc-GM3) has been found to function as K99 receptor on horse erythrocytes [5]. This was confirmed by recent studies demonstrating that NeuGc-GM3 [6, 7] and also *N*-glycolylsialoparagloboside (NeuGc-SPG) [6] were potential receptors of the K99 lectin in the pig small intestine. These conclusions were supported by data on the inhibition of human erythrocytes agglutination using intact bacteria and a series of synthetic neuraminic acid derivatives [8].

A few lectins specific for sialic acid were described, most of them exhibiting greater affinities for *N*-acetylneuraminic acid-containing glycoconjugates [9]. Interestingly K99 is a sialic acid specific lectin that exhibits a much higher affinity for *N*-glycolylneuraminic acid-containing glycolipids, compared to NeuAc containing ones [6–8, 10]. Hence, K99 lectin could be an interesting tool for visualizing NeuGc containing glycoconjugates. Studies of receptor-active glycolipids for K99 lectin were carried out either with ^{125}I [6], ^{35}S [7] or ^{14}C labelled [11] intact bacteria. Binding of isolated K99 fimbriae to glycoproteins from bovine and porcine colostrum was studied using rabbit anti-fimbriae IgG in combination with biotin-conjugated anti-rabbit IgG antibodies [12]. Recently, binding of fluorescein isothiocyanate (FITC) labelled K99 fimbriae to cryostat sections of pig small intestine was detected and demonstrated to be due to the intestinal mucin glycopeptides [13, 14].

In the present study, we describe the synthesis of biotinylated derivatives of K99 lectin (CbioK99), able to recognize isolated receptors in an overlay binding assay as well as receptors localized on the surface of HeLa cells. We demonstrate that CbioK99 is a versatile probe since it can be detected by various streptavidin conjugates.

* To whom correspondence should be addressed.

Materials and methods

Materials

Escherichia coli strain B80 (O20:K17?:K99) originated from J. A. Morris, Central Veterinary Laboratory, Weybridge, Surrey, UK. HeLa cells (ATCC CCL2) were obtained from Flow Laboratories, UK.

The following materials were obtained from the sources indicated: yeast extract from Difco, USA; biocytin hydrazide from Molecular Probes, USA; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, bovine serum albumin fraction V from Sigma, USA; Ultrogel AcA 202 from IBF, France; Nitroplus membrane 0.22 μm from Micron Separations, USA; streptavidin–alkaline phosphatase conjugate, fluorescein–streptavidin, [^{35}S]streptavidin (654 Ci mol^{-1}) and Hyperfilm™ MP from Amersham International, UK; nitroblue tetrazolium and bromochloroindolyl phosphate from Gibco-BRL, USA; Fluoprep, penicillin, streptomycin and foetal calf serum from Biomérieux, France; *Vibrio cholerae* neuraminidase (EC 3.2.1.18) from Boehringer, Germany; tissue culture chamber slides from Nunc, USA; poly(isobutyl methacrylate) from Röhm, Germany. Molecular weight standards were from Pharmacia, Sweden and HPTLC Silica Gel 60 pltes from Merck, Germany.

Purification of K99 lectin

The bacteria were grown on solid minimal casein medium (Minca medium) [15], supplemented with 1 g l^{-1} of yeast extract, 18 h at 37 °C, harvested and washed in 150 mM phosphate buffered saline (PBS), pH 7.2, resuspended in the same buffer (A_{700} = 50), heated for 20 min at 65 °C and centrifuged (12 000 × *g*, 30 min). The supernatant was adjusted to pH 4.8 and incubated at 37 °C for 2–3 h until a frothy precipitate appeared. The pellet collected by centrifugation (12 000 × *g*, 30 min) was resuspended in 150 mM PBS, stored overnight at −20 °C then thawed at room temperature (cryoprecipitation). The resulting precipitate was resuspended in the same buffer and dialysed against 3 × 500 vol. of 0.9% (w/v) NaCl. Protein content was measured according to the method of Lowry *et al.* [16] using bovine serum albumin (BSA) as a standard. The lipopolysaccharide (LPS) from the same strain was extracted using the phenol–water procedure [17].

Biotinylation of the lectin via carboxylic groups

Biocytin was coupled to carboxylic groups using an approach described earlier [18]. A typical reaction was carried out with 10 mg of protein (0.61 μmol) in 45 ml 0.9% NaCl (adjusted at pH 4.75 with HCl) and various concentrations of biocytin hydrazide (BCHZ). The reaction was initiated by addition of 610 μmol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 5 ml of 0.9% NaCl. The pH was maintained at 4.75 by addition of 10 mM HCl with a pH-stat, type 655 (Metrohm). Aliquots (2.5 ml) were withdrawn at predetermined intervals and added to 2.5 ml 1 M sodium acetate buffer, pH 4.75, to quench the reaction with carbodiimide. Excess reagents were removed on an Ultrogel AcA 202 column (1.5 × 30 cm) equilibrated in 0.9% NaCl. The amount of biocytin linked to the lectin was determined by a spectrophotometric method using hydroxyazobenzoic acid [19]. Recovery of biocytin-K99 was 85%. Haemagglutination was performed in 10 mM PBS, pH 7.2, using 50 μl of a suspension of 2% (w/v) washed horse erythrocytes. The titre, i.e., the highest dilution still giving obvious haemagglutination, was determined in serial twofold dilutions (50 μl) of the biotinylated lectin (300 μg ml^{-1}) in microtitre trays, after incubation for 2 h at 4 °C.

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting

20% SDS-PAGE was performed using a Phast System™ apparatus (Pharmacia, Sweden). The sample buffer was 10 mM Tris-HCl, pH 8.0, containing 2.5% SDS and 5% β-mercaptoethanol (by vol). Some gels were silver stained [20] for detection of proteins and lipopolysaccharide. After electrophoresis, the biotinylated proteins were transferred to a Nitroplus membrane, 0.22 μm, with a semi-dry transfer system (Ancos, Denmark) as described [21]. The membranes were saturated with 2% BSA in 50 mM PBS, pH 7.2, 0.1% Tween 20 for 1 h at room temperature and incubated with an appropriate dilution of streptavidin–alkaline phosphatase conjugate in 100 mM Tris HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 0.05% Tween 20. The membranes were washed three times and colour development was performed, at room temperature, in the dark, with nitroblue tetrazolium and bromochloroindolyl phosphate in the same buffer [22].

Bacterial adhesion to HeLa cells

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM), containing 4 mM glutamine and supplemented with 1U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% (by vol) of decomplemented foetal calf serum. The cells were incubated in a 5% CO_2 atmosphere. For bacterial adhesion, HeLa cells were cultured on a tissue culture chamber slide, and washed with DMEM before use. *E. coli* B80, grown at 37 °C, were suspended in DMEM and incubated (500 μl, A_{700} = 0.1) with subconfluent cells for 30 min at 37 °C. Unbound bacteria were removed by extensive washing (five times) then the cells were fixed with methanol and Giemsa stained. As negative control, *E. coli* B80 grown at 18 °C, a non-permissive temperature for expression of K99 fimbriae [23], were incubated under the same conditions. For adhesion inhibition test, purified lectin was added in excess (500 μg per well).

Binding of biotinylated K99 lectin to HeLa cells

HeLa cells were cultured on tissue culture chamber slides and washed twice with DMEM. Biotinylated lectin (25 μg in 500 μl DMEM) was added, and the cells were incubated 1 h at 37 °C. Control wells received DMEM without

biotinylated lectin. The wells were washed three times and incubated 30 min at 37 °C with fluorescein–streptavidin at 1:2000. The cells were washed three times, submerged with a thin layer of Fluoprep (antifading agent), covered with slide cover slips and submitted to fluorescence microscopy (type BH-2, Olympus). As a control, the receptor sites were saturated with the native purified lectin (500 μg per well) prior to the CbioK99 addition. Cells were also desialylated with *Vibrio cholerae* neuraminidase as previously described [24].

Chromatogram-binding assay

Ganglioside NeuGc-GM3 was extracted from horse erythrocytes as described [11]. All other gangliosides were from Sigma. Sialic acid content was estimated by a colorimetric assay [25]. Various amounts of gangliosides were chromatographed on aluminium-backed silica gel 60, high performance thin layer chromatography (HPTLC) plates developed in chloroform–methanol–0.2% (w/v) $CaCl_2$ in water (60:35:8 by vol). The plates were coated with 0.1% (w/v) poly(isobutyl methacrylate) for 1 min, soaked in 10 mM PBS–2% (w/v) BSA for 2 h at room temperature and overlaid with CbioK99 (50 μg ml^{-1}) in PBS–0.2% (w/v) BSA for 4 h at room temperature or overnight at 4 °C. The plates were washed three times with cold PBS and incubated with [^{35}S]streptavidin (2×10^6 counts min^{-1} ml^{-1}) for 2 h at room temperature. The plates were washed five times with cold PBS, air dried and autographed using Hyperfilm™ MP. For neutral sugars visualization, glycolipids were stained by an orcinol–sulfuric acid reagent [0.1% (w/v) orcinol in 20% (v/v) sulfuric acid].

Results and discussion

Purification of the K99 lectin

The major problem encountered in the purification of K99 fimbriae, is the contamination by outer membrane components, which are difficult to remove due to their hydrophobic binding to the fimbriae. This is especially true for LPS which, in addition, cannot be detected by Coomassie blue staining, generally used for this purpose [26–28]. During the first attempts of K99 lectin biotinylation, carried out with preparations obtained according to earlier purification procedures, a precipitate appeared, demonstrated to be due to the presence of LPS. Indeed, the same phenomenon was observed when mixing lysozyme with LPS in equivalent conditions. We conclude that the coprecipitation, observed at pH 4.8, was due to interactions between the acidic LPS and K99 lectin (or lysozyme) having an isoelectric point ranging between 10 and 11. Taking advantage of this observation, a precipitation step at pH 4.8 was added to the purification procedure and analysis by SDS-PAGE showed that the precipitate was simultaneously enriched with K99 lectin and LPS (Fig. 1a, lane 4). This precipitate was redissolved in PBS, frozen and thawed at

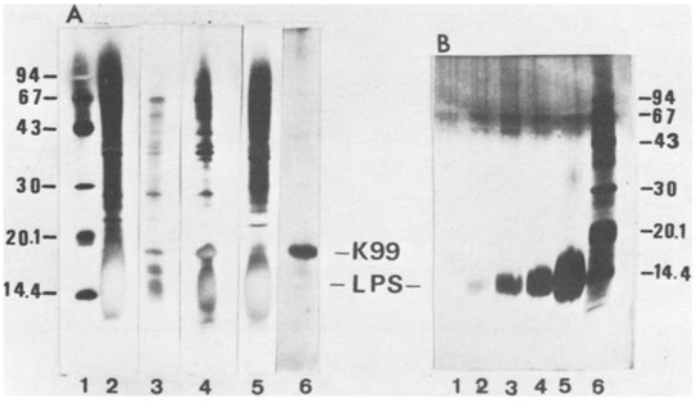

Figure 1. Electrophoretic control of K99 purification steps. (a) Purification of K99 lectin. Samples were separated on 20% polyacrylamide gel and silver stained. Lane 1, molecular weight standards (kDa), from top to bottom: phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lactalbumin. Lane 2, heat shock extract. Lanes 3 and 4, supernatant and pellet of precipitation at pH 4.8, respectively. Lanes 5 and 6, supernatant and pellet of cryoprecipitation. (b) Threshold detection of lipopolysaccharide isolated from *E. coli* B80. Lanes 1, 2, 3, 4 and 5 represent 1, 3, 10, 30, and 100 ng of LPS, respectively. Lane 6, molecular weight standards.

room temperature to give a single protein with an M_r of 18 kDa (Fig. 1a, lane 6), while LPS was found in the supernatant (Fig. 1a, lane 5).

For further studies, silver staining was used to verify that the purified lectin was LPS-free. LPS purified from the same strain gives a diffuse band migrating as a 14 kDa compound, and can be detected at the level of 3 ng (Fig. 1b). For the preparation showed Fig. 1a, lane 6 (300 ng of protein), LPS represented less than 1%.

Biotinylation of the K99 lectin via carboxyl groups

Biotinylation of proteins is usually carried out via ε-NH_2 groups of lysine [29]. This strategy is not suitable for K99 lectin since lysine residues were localized in the receptor binding domain of the K99 subunits [30–32]. To circumvent a similar problem, Rosenberg *et al.* [33] have biotinylated nerve growth factor (NGF) via carboxyl groups using biotin hydrazide and a water soluble carbodiimide. We employed the same approach to label the K99 lectin but we used biocytin hydrazide. Rosenberg *et al.* [33] studied the effect of EDC concentration on the biotinylation of NGF in buffered media. We gave more attention to the nucleophile (BCHZ) concentration and used an unbuffered medium to let us record the kinetics of the reaction.

Estimation of the incorporated biocytin residues, from the HCl taken up [18], shows good correlation (correlation coefficient = 0.993) with the data obtained by the colorimetric assay (Fig. 2). The course of the reaction was established for three BCHZ/K99 (ϕ) ratios (Fig. 3). After 20 min, five biocytin residues were incorporated at $\phi = 5000$,

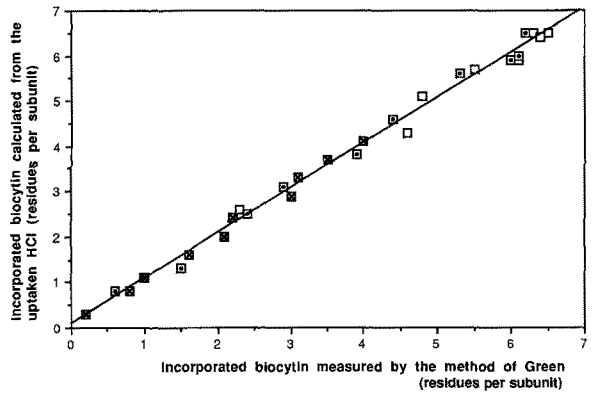

Figure 2. Correlation between the incorporated biocytin residues calculated from the HCl taken up, and measured by the colorimetric assay of Green [17]: □, $\phi = 5000$; ⊠, $\phi = 500$; ⊡, $\phi = 250$.

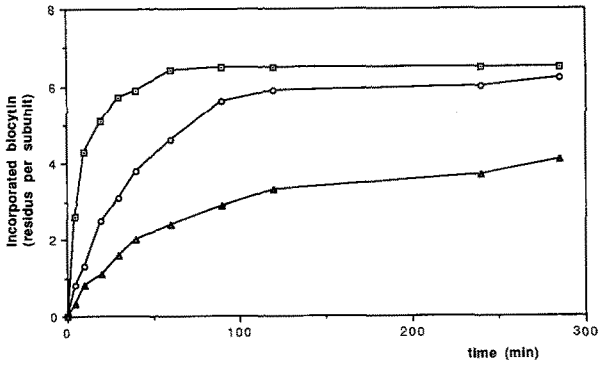

Figure 3. Kinetics of biocytin incorporation in K99 subunits as a function of the BCHZ/K99 (ϕ) ratio: □, $\phi = 5000$; ○, $\phi = 500$; △, $\phi = 250$.

while 2.4 and 1.1 residues were incorporated for $\phi = 500$ and 250, respectively.

The reaction products were separated by electrophoresis, transferred to nitrocellulose and revealed by straptavidin–alkaline phosphatase conjugate (Fig. 4). When the coupling reaction was carried out at $\phi = 500$ or 250, covalently crosslinked dimers were observed (Fig. 4b, c). For $\phi = 100$ even trimers were seen (Fig. 4a). Since lysine and arginine residues possess a nucleophilic power, a possible parasite reaction occurs on the activated carboxylic groups, favoured by low BCHZ concentrations and by longer incubation, resulting in the formation of interchain covalent linkages.

This has a direct incidence on the haemagglutinating activity of CbioK99, as tested on horse erythrocytes (Table 1). ϕ was critical for preserving the haemagglutinating activity. At $\phi = 5000$, fixation of 2.5 biocytin residues per subunit lead to a decline of the residual haemagglutinating titre (RHT) from 256 to 64 (Table 1) while, at the same biotinylation degree obtained at lower ϕ values, RHT was unchanged. High concentrations of BCHZ ($\phi > 500$) resulted in the labelling of less accessible carboxyl groups

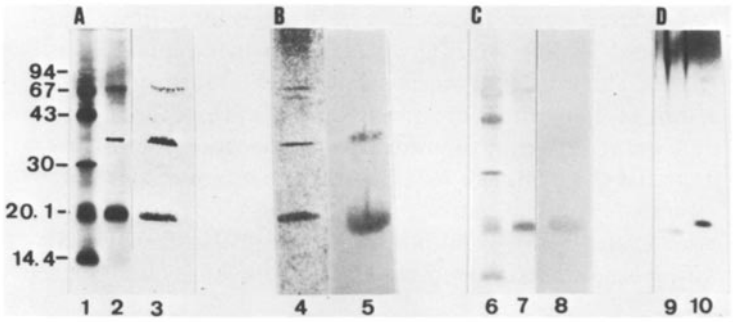

Figure 4. Electrophoretic control and blotting analysis of CbioK99. K99 lectin was biotinylated at various BCHZ/K99 (ϕ) ratios: (a) $\phi = 100$, 180 min; (b) $\phi = 250$, 60 min; (c) $\phi = 500$, 20 min; and (d) $\phi = 5000$, 5 min. Lanes 1 and 6, molecular weight standards as in Fig. 1. Lanes 2, 4, 7 and 10, silver stained gels of the reaction products. Lanes 3, 5 and 8 represent the blotting analysis of the samples run in lanes 2, 4 and 7, respectively. Lane 9, native K99 lectin.

Table 1. Evolution of the residual haemagglutination titre (RHT) of K99 lectin during the biotinylation reaction compared to the number of incorporated[a] biocytin (BC) residues.

ϕ at the beginning of the reaction					
$\phi = 5000$		$\phi = 500$		$\phi = 250$	
BC	*RHT*	*BC*	*RHT*	*BC*	*RHT*
0	256	0	256	0	256
2.6	64	0.8	256	0.3	256
4.3	32	1.3	128	0.8	256
5.1	0	2.5	128	1.1	256
5.7	0	3.1	64	1.6	256
		3.8	32	2	256
		4.6	4	2.4	128
		5.6	0	2.9	128
		5.9	0	3.3	64
				3.7	32
				4.1	16

[a] ϕ is the BCHZ/K99 ratio.

that probably modified the spatial organization of the fimbriae. Low BCHZ concentrations ($\phi < 250$) have the disadvantage of forming covalently linked di- and trimers (see above), leading to loss of haemagglutinating activity (Fig. 4a). Biotinylation has an incidence on the M_r of K99 subunits, since CbioK99 migrate at 21 kDa instead of 18 kDa for the native K99 lectin (Fig. 4d).

Using the same approach to modify carboxyl groups of K99 with glycine methyl ester, Jacobs *et al.* [31] demonstrated that modification of five out of the nine carboxyl groups did not alter the haemagglutinating activity but rather increased it twofold. Our results indicate that a maximum of two residues per subunit can be modified without altering

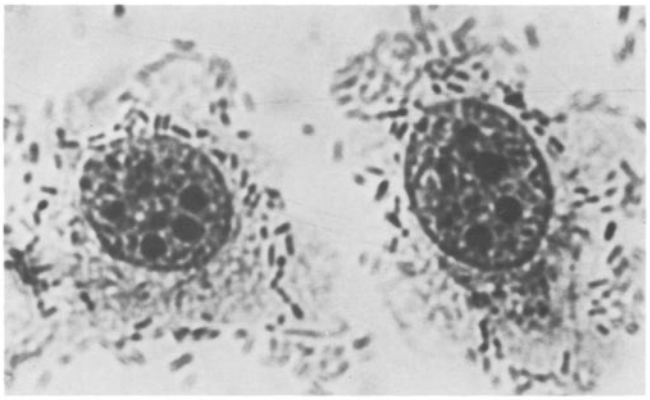

Figure 5. Adhesion pattern of *E. coli.* B80 to HeLa cells. HeLa cells were cultured on a tissue culture chamber slide and incubated with the bacteria 30 min at 37 °C. The cells were washed to remove unbound bacteria, Giemsa stained and observed under the light microscope at a magnification of × 1000.

the RHT. This difference can be explained by a different spatial organization of the lectin with the incorporated biocytin residues compared with glycin methyl ester. For further studies, biotinylation was carried out at $\phi = 250$, for 40 min, in order to obtain two biocytin residues per subunit.

Adhesion of E. coli carrying K99 fimbriae and CbioK99 to the surface glycoconjugates of HeLa cells

E. coli B80 adhere to the HeLa cells (Fig. 5) and this seems due to K99 lectin, since this phenomenon is not observed for the same strain cultured at 18 °C, a temperature which does not allow K99 fimbriae expression. Recent reports introduced cell models to study human enteropathogenic *E. coli.* Various adhesion patterns were determined with the cell lines used (HeLa [34, 35]; Hep2 [36]; and HT29 [37]) and have been established as diffuse, localized [38] or aggregative adhesion [36]. The adhesion of *E. coli* B80 shows a diffuse adhesion pattern since the bacteria cover the entire cell surface (Fig. 5). Adhesion of CbioK99, as shown by fluorescence microscopy, was equivalent to the one of the *E. coli* B80 (Fig. 6). Sialic acid residues were responsible for adhesion since neuraminidase treatment dramatically decreased the CbioK99 recognition as well as the adhesion of intact bacteria.

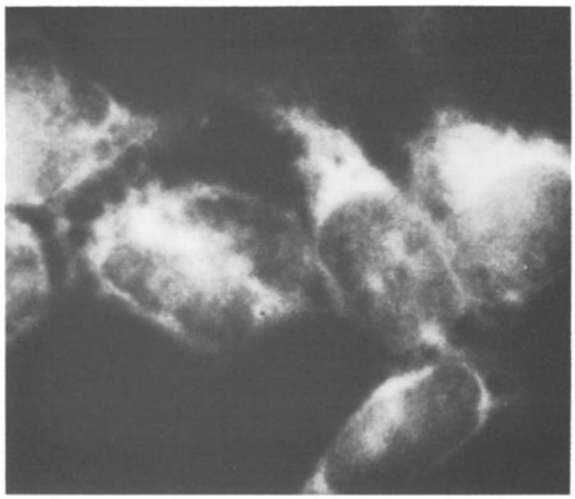

Figure 6. Adhesion of CbioK99 to HeLa cells. HeLa cells were grown on a tissue culture chamber slide and incubated with 25 μg ml^{-1} of CbioK99, 1 h at 37 °C. After washing, the attached CbioK99 was revealed with FITC–straptavidin conjugate and observed under the fluorescence microscope at a magnification of × 1000.

Chromatogram-binding assay

Previous studies, based on recognition of isolated glycolipids by radiolabelled K99-carrying bacteria, reported that NeuGc-GM3 was a K99 receptor on horse erythrocytes [5] and, with NeuGc-SPG, was responsible for the susceptibility of young animals to infection with K99-carrying ETEC [6, 7]. To be sure that K99 lectin has the same recognition specificities after biotinylation, CbioK99 binding was tested on reference gangliosides. As the binding assay was carried out on aluminium-backed silica gel, the resorcinol–HCl spray, revealing sialic acids, was not advisable because hydrochloric acid destroys the aluminium sheet. Thus, the plate was revealed with an orcinol–H_2SO_4 spray (Fig. 7a) (see previous page) and this explains the staining differences of the standard glycolipids containing relatively equivalent amounts of sialic acid but not neutral sugars. The biotinylated lectin specifically binds to NeuGc-GM3 (Fig. 7b). Bacteria carrying K99 lectin recognized NeuGc-GM3 at a minimal range of 100 ng [6]. The CbioK99 probe was equally sensitive since it was able to detect 90 ng (calculated from the sialic acid content) of the same ganglioside (Fig. 7c).

Biotinylated K99 lectin offers a wide range of detection techniques, since the streptavidin (or avidin) can be coupled to several detectable molecules and avidin–biotin technology permits several strategies [39]. Applications of lectins, either in solution or, commonly, under immobilized form, for detection and purification of variety of carbohydrate-containing components, have been reviewed by Lis and Sharon [40]. CbioK99 will be a tool for the detection of specific receptors for K99-carrying ETEC. It can also be an additional probe for the detection of Haganitziu Deicher antigens (e.g., HD3 = NeuGc-GM3) interestingly found in malignant tissues and sera from humans with various tumours [41, 42].

Acknowledgement

Dr Yves Cenatiempo is thanked for reading the manuscript critically.

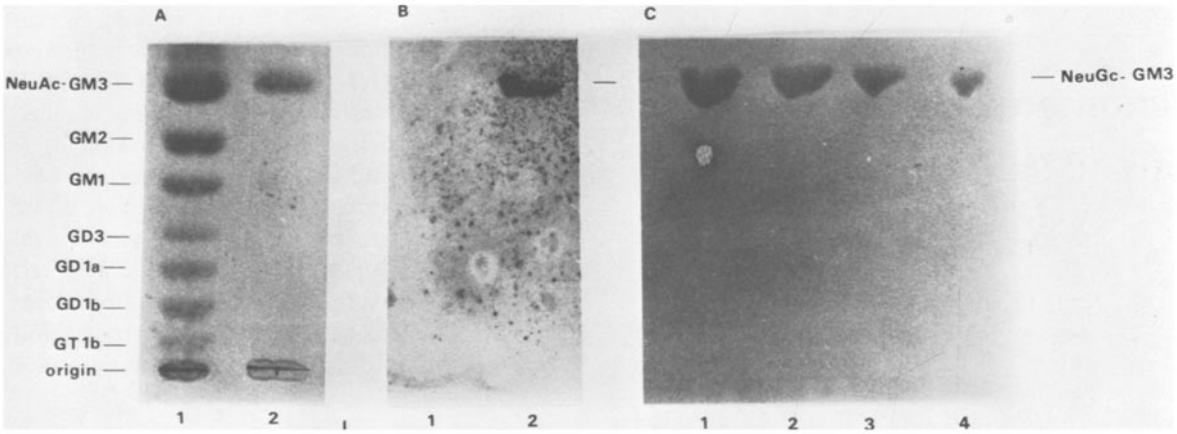

Figure 7. Binding of CbioK99 to standards glycolipids. The glycolipids were separated by thin layer chromatography and (a) revealed with an orcinol–sulfuric reagent or (b) overlaid with CbioK99. Amounts of standard glycolipids used (calculated from sialic acid content) were: lanes 1, 250 ng GT1b, GD1a, GD1b and GD3, 500 ng GM1 and GM2, 750 ng NeuAc-GM3; lanes 2, 500 ng NeuGc-GM3. (c) Threshold detection of NeuGc-GM3 with CbioK99. Lanes 1, 2, 3 and 4 represent 200, 100, 50 and 25 ng of sialic acid content, respectively.

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