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Two strains of the Madin–Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions

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The glycosphingolipids (GSLs) of two sublines of Madin–Darby canine kidney (MDCK) cells, an epithelial cell line, were characterized by t.l.c., antibody overlay and mass spectrometry. The major characteristic which distinguishes the two MDCK cell strains is their trans-epithelial electrical resistance which is typically of the order of 3000 ohm.cm^2 for strain I and 100 ohm.cm^2 for strain II cells. Strain I and II cells were equally rich in glycolipids, the cellular GSL/phospholipid ratio being 0.04. However, while the phospholipid patterns were identical, the GSLs showed striking differences, and each cell strain expressed appreciable amounts of GSLs that were not found in the other strain. Both cell types possessed neutral GSLs with one, two or three carbohydrate moieties. The monoglycosylceramide accounted for 50% of the total GSLs in each strain. However, while in strain I cells over 90% of this monoglycosylceramide was monoglucosylceramide, in strain II cells over 90% consisted of monogalactosylceramide. In addition, MDCK strain II cells selectively expressed GSLs belonging to the globo series (26% of its neutral GSLs), including globoside and Forssman antigen, a globoside derivative. MDCK strain I cells, on the other hand, expressed another series of GSLs with 4–7 carbohydrate moieties characterized by the common sequence Hex-HexNAc-Hex-Hex-Cer. The presence of two fucosylated GSLs in these series was established. Both MDCK strain I and II cells contained negatively charged GSLs, the major component of which was the ganglioside GM3. MDCK strain II cells in addition expressed sulfatide, the sulfated derivative of galactosylceramide. The present findings further demonstrate that the MDCK strain I and II cells are different cell types which either are derived from different segments of the distal part of the nephron or represent different stages in the differentiation of the same cell type.

Key words: glycosphingolipids/Madin–Darby canine kidney cells/characterization

Introduction

In most mammalian cells the large majority of the glycolipids consists of glycosphingolipids (GSLs) (Ishizuka and Yamakawa, 1985). GSLs have been reported to occur mainly in the plasma membrane where they appear to be localized in the non-cytoplasmic leaflet (Op den Kamp, 1979). GSLs show a remarkable variation between species, individuals, tissues and cells (Hakomori, 1981; Breimer *et al.*, 1981a, 1982). They undergo stage-specific changes during development. GSLs have been used as markers to identify cells both in embyros and in the adult organism (Raff *et al.*, 1978; Hakomori and Kannagi, 1983; Feizi, 1985). So far, the function of the individual GSL species is essentially unknown (Thompson and Tillack, 1985; Makita and Taniguchi, 1985).

With respect to GSL distribution, epithelial cells are especially intriguing. The cell surface of these cells is polarized into two plasma membrane domains, separated by junctional complexes (for review, see Simons and Fuller, 1985). The apical plasma membrane facing the lumen of the tissue has a protein and lipid composition different from the basolateral membrane which is in contact with the internal milieu, facing the mesenchymal space and the blood supply. The lipid composition of the plasma membrane domains has been best studied in epithelial cells from the small intestine (Forstner and Wherrett, 1973; Kawai *et al.*, 1974; Brasitus and Schachter, 1980; Hansson, 1983). Their GSLs are enriched in the apical membrane to such an extent that if they were specifically localized in the non-cytoplasmic leaflet of the membrane, this leaflet would be almost exclusively GSL (van Meer *et al.*, 1985). In some other epithelial cell types sphingomyelin instead of GSLs appears to be concentrated in the apical membrane (Chapelle and Gilles-Baillien, 1983; Hise *et al.*, 1984; Meier *et al.*, 1984; Molitoris and Simon, 1985).

We are using the Madin–Darby canine kidney (MDCK) cell line as an experimental model to study the generation and maintenance of cell surface polarity in epithelial cells (Simons and Fuller, 1985). There are two strains of this cell line which originate from the kidney of a cocker spaniel (Gaush *et al.*, 1966). The strain I cells are derived from an early passage, whereas the strain II cells predominate in later passages (Barker and Simmons, 1981; Richardson *et al.*, 1981; Valentich, 1981). Both of these strains are thought to be derived from the distal tubule or collecting duct of the nephron (McRoberts *et al.*, 1981; Valentich, 1981; Ojakian and Herzlinger, 1984; see however Richardson *et al.*, 1981 for an alternative opinion concerning MDCK strain II cells). The cell of origin is not known and is difficult to determine since it is probable that the cells have undergone changes in continuous culture. The most dramatic difference between these strains is their electrical resistance. The strain I cells form 'tight epithelia' with resistances over 3000 ohm.cm^2, whereas the strain II cell layers are 'leaky' with 100 ohm.cm^2 (Richardson *et al.*, 1981; Balcarova-Ständer *et al.*, 1984). The morphology of these cells is also different. Strain I cells are non-ciliated, have fewer microvilli on their apical surfaces and a more folded lateral membrane in comparison with strain II cells which are ciliated and columnar in shape when grown on permeable supports (Valentich, 1981; Barker and Simmons, 1981; von Bonsdorff *et al.*, 1985).

The protein and lipid compositions of the apical and basolateral membrane in filter-grown MDCK I and II cells have not yet been fully characterized but their plasma membranes appear to be as polarized as epithelial cell surfaces *in vivo* (Simons and Fuller, 1985). The phospholipid composition of the apical membranes is almost identical in MDCK I and II cells but dramatically different from that of the basolateral membrane. Again, the phospholipid composition of the basolateral membrane is similar in

the two strains (van Meer and Simons, 1982; van Meer *et al.*, 1985). These differences were determined by analysing the phospholipid compositions of viruses budding from either the apical or the basolateral membranes of the cells. The viruses used are known to incorporate into their envelopes the lipids present in the plasma membranes from which they bud. In a comparative study, the same viruses budding from non-polarized BHK cells were found to have identical phospholipid compositions (van Meer *et al.*, 1985).

As a prelude to analysing the distribution of GSLs in the MDCK strain I and II cells we determined the total GSL composition of these two cell strains and discovered dramatic differences between the MDCK strain I and II GSLs. Here we report these analyses from t.l.c., antibody analysis and mass spectrometry.

Results

The overall lipid composition of the two sublines MDCK strain I and MDCK strain II grown on nitrocellulose filters were very similar (Table I). However, when the GSLs were purified and analysed by t.l.c. strikingly different patterns were revealed (Figure 1). The GSL patterns of cells grown on filters were identical to those of cells grown in tissue culture flasks which were used for initial characterization of the components. However, the amount of ganglioside as compared with phospholipid was about five times higher in the cells from the tissue culture flasks.

The GSLs can be divided into two classes, the neutral GSLs and the acidic GSLs. The acidic GSLs carry one or more negative charges at neutral pH in the form of sialic acid (gangliosides) or sulfate (sulfatides). The acidic GSLs of MDCK cells (Figure 1B) were easily identified. The dominant ganglioside in the chromatogram of both cell strains (Figure 1B band n) co-migrated with GM_3 (hematoside) as previously described (Markwell *et al.*, 1984). The sialic acid (N-acylneuraminic acid) of the GM_3 in

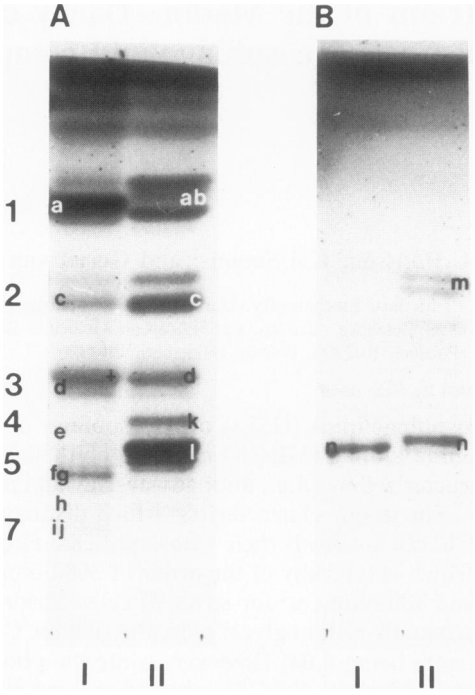

Fig. 1. Thin-layer chromatogram of the neutral **(A)** and acid **(B)** GSLs prepared from MDCK strain I (left lane) and MDCK strain II (right lane) cells grown on plastic supports. The fractions were prepared as described in Materials and methods and were analysed by h.p.-t.l.c. using chloroform/methanol/water 60:35:8 (v/v) as a solvent. The bands were visualized by the anisaldehyde reagent and all bands were green as for sugars except the + marked band. 1/50 and 1/80 of the neutral GSL fractions and 1/250 and 1/200 of the acid GSL fractions from 10^9 MDCK strain I and II cells were applied, respectively. The letters refer to the individual GSLs shown in Table II. The numbers on the left refer to the numbers of sugars.

Table I. Lipid composition of MDCK strain I and II cells grown on nitrocellulose filters[a]

	MDCK I		MDCK II	
	nmol/filter	%	nmol/filter	%
Phospholipids[b]	109 ± 9		131 ± 9	
Phosphatidylcholine		53.1 ± 0.4		52.5 ± 0.6
Phosphatidylethanolamine		25.7 ± 0.4		28.3 ± 0.6
Phosphatidylinositol		7.7 ± 0.1		5.8 ± 0.2
Phosphatidylserine		6.0 ± 0.3		4.4 ± 0.3
Sphingomyelin		7.6 ± 0.3		9.5 ± 0.2
Cholesterol	37.3 ± 0.6		39.0 ± 5.0	
Free cholesterol		82		79
Cholesterolester		18		21
Glycosphingolipids[c]	4.7 ± 1.0		4.6 ± 0.8	
Neutral		85		85
Acidic		15		15

[a]The total lipids were purified and analysed as described in Materials and methods.
[b]The numbers represent the mean composition of three monolayers of cells on a filter and are followed by the sample standard deviation (n=3). For the number of cells per filter see Materials and methods.
[c]The values for the total concentration of glycosphingolipids are the mean of two preparation protocols used and are followed by the difference of their mean. The lower values are from the protocol including SDS-solubilization.

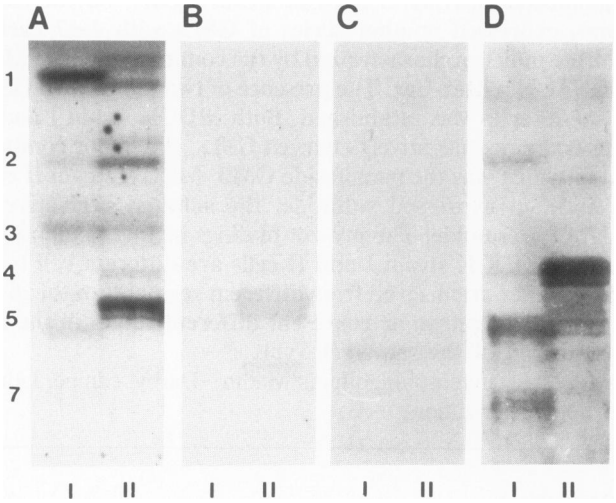

Fig. 2. The neutral GSL fractions of MDCK strain I and II cells grown on plastic supports were analysed after separation by h.p.-t.l.c. by reaction with a monoclonal anti-Forssman antibody **(B)**, a monoclonal anti-blood group H type 2 antibody **(C)** and serum from a woman with blood group p^k **(D)**. Identical fractions, chromatographed at the same time, were visualized by the anisaldehyde reagent **(A)**. The same solvent as in Figure 1 was used. The antibodies bound were detected by ^{125}I-labeled anti-mouse Ig antibodies and for D with ^{125}I-labeled anti-human IgG antibodies, followed by autoradiography for 4 days. 1/100 and 1/160 of each fraction from 10^9 MDCK strain I and II cells were used, respectively.

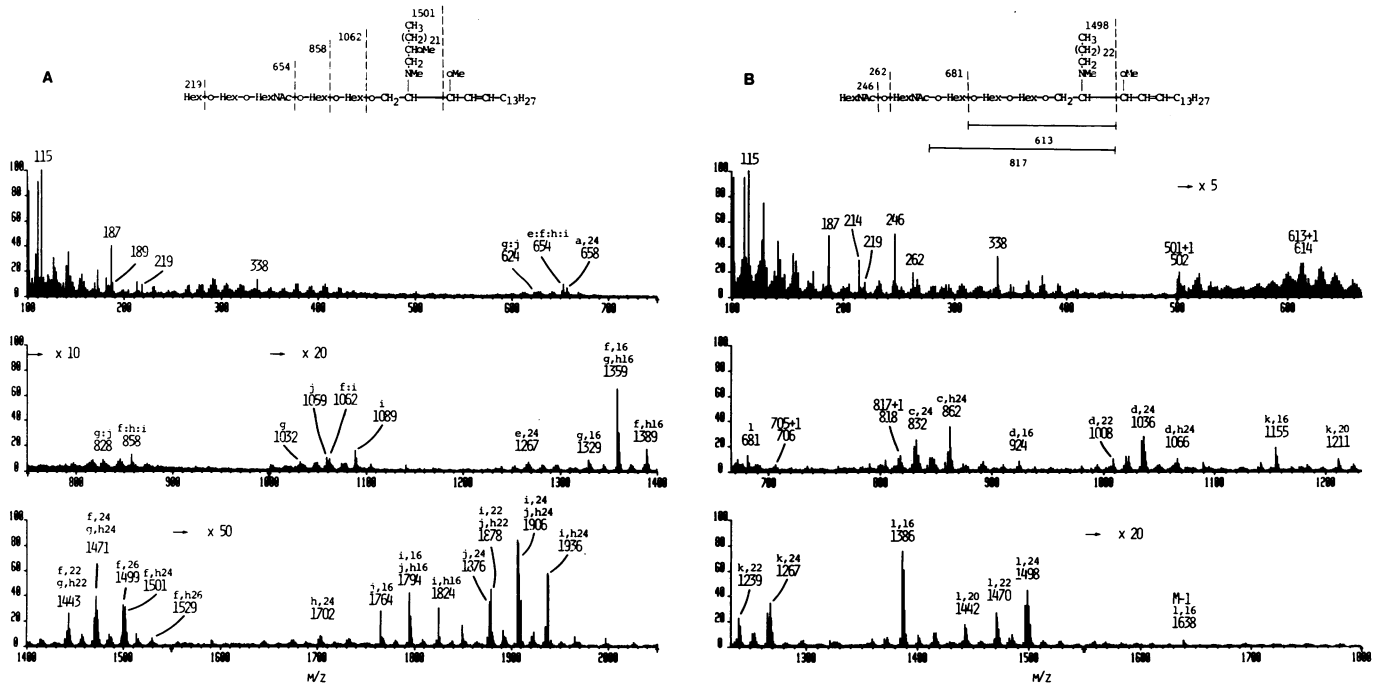

Fig. 3. Mass spectra of neutral GSLs from MDCK strain I **(A)** and MDCK strain II **(B)** cells. A total mixture of neutral GSLs from each MDCK strain I and MDCK strain II cells was permethylated and part of each also $LiAlH_4$-reduced. The reduced and non-reduced derivatives were analysed separately. About 50 μg of a sample was inserted via the direct inlet probe to an 'in-beam' position and the ion source was heated from 180°C to achieve a fractional evaporation. One example from each cell line is shown recorded at 305°C for MDCK strain I cells and at 280°C for MDCK strain II cells of the permethylated-reduced derivative. The letter code in the spectra refers to the individual GSLs shown in Table II and discussed in the text. The formulas at the top are included as an example of the fragmentation pattern and represent one characteristic component in each cell line. The masses shown are nominal, i.e. obtained by counting paper spectra manually.

MDCK cells was shown to be N-acetylneuraminic acid by t.l.c. in a solvent system that separates N-acetyl GM_3 from N-glycolyl GM_3 (Ångström *et al.*, 1981). Trace amounts of more slowly moving gangliosides were also present. The major difference between the two cell lines was that sulfatide (galactosylceramide-sulfate, band m in Figure 1B) which has been demonstrated in MDCK cells by Ishizuka *et al.* (1978), was found to be present only in MDCK strain II cells. It runs as a double band due to differences in the lipophilic moiety.

The bands found in the neutral GSL chromatogram (Figures 1A and 2A) were further characterized by antibody overlay using antibodies with a specificity for defined GSLs (Magnani *et al.*, 1982; Hansson *et al.*, 1983). Various neutral GSL bands showed reactivity (Figure 2) with monoclonal antibodies against the Forssman antigen (lane B), human blood group H type 2 chain (lane C) and against an antiserum from a pregnant woman of blood group p^k, containing antigloboside antibodies (lane D; Hansson *et al.*, in preparation). No reaction was observed with monoclonal antibodies against blood groups A, B, X, Le^b and H type 1 (for specificities see Makita and Taniguchi, 1985). The chromatograms of acidic GSLs (Figure 1B) did not bind any of the antibodies. The GSLs which were recognized by the various antibodies in MDCK strain I cells were completely different from those recognized in MDCK strain II (Figure 2B–D). An interpretation of the antibody binding is given below.

A more direct approach to identify the structure of the neutral GSLs of each cell strain was analysis of the permethylated and permethylated-$LiAlH_4$-reduced GSL derivatives on a mass spectrometer (Breimer *et al.*, 1979, 1981a). Examples of mass spectra of the permethylated-reduced samples recorded at higher temperatures and thus containing the larger structures are shown in Figure 3. An integration of the mass spectra, antibody binding and t.l.c. data is presented in Table II. This table shows the GSL structures assigned to each cell line, their relative amounts, the major fatty acids found in the individual GSL species and the major peaks obtained from the mass spectra.

On the chromatogram of the MDCK strain II neutral GSLs, the monoclonal anti-Forssman antibody (Figure 2, lane B) reacted strongly with a band in the 5-sugar interval and more weakly with a band in the 7-sugar interval. Mass spectrometry of the permethylated-reduced sample (Figure 3B) showed a series of peaks for the carbohydrate–fatty acid-containing fragments (the F-fragments; Breimer *et al.*, 1979), from *m/z* 1386 for the 16:0 fatty acid to *m/z* 1498 for the 24:0 fatty acid, characteristic of the Forssman antigen containing five sugars. Sequence ions containing different numbers of sugars from the non-reducing end were found in both the permethylated and permethylated-reduced derivatives (see Table II). Identical peaks were found in the mass spectra of a purified Forssman antigen (Karlsson *et al.*, 1974a). The combined information suggests the structure of the MDCK neutral GSL which reacted with the Forssman antibody shown in Table II (1). It is identical to the structure of the Forssman hapten reported by Siddiqui and Hakomori (1971). The four sugar components of the MDCK strain II cells (k, Table II) showed peaks in mass spectra (Figure 3) identical to peaks recorded for globoside. Its identity was confirmed by the fact that it co-migrated with a globoside reference and that it reacted strongly with a serum containing anti-globoside antibodies (Figure 2, lane D). The di- and triglycosylceramides (c and d, Table II) of MDCK strain II cells contained only hexoses (based on the mass

Table II. Glycosphingolipid composition of MDCK strain I and II cells. The table also includes major peaks found in mass spectra supporting the individual components

Probable glycosphingolipid[a] sequences	MDCK I				MDCK II			
	Estimated[b] amount	Major[c] F-fragments (*m/z*)	Major[d] sequence ions (*m/z*)	Fatty acid[e] composition	Estimated amount	Major F-fragments (*m/z*)	Major sequence ions (*m/z*)	Fatty acid composition
Neutral glycosphingolipids								
a. Glc-Cer	>56%	516,546,		16,h16,h24	<5%	516,546		16,h16
b. Gal-Cer	<6% 62%	658			>47% 52%	628,658		24,h24
c. Hex-Hex-Cer	18%	720,832,862		16,24,h24	13%	720,750,832,862		16,h16 24,h24
d. Hex-Hex-Hex-Cer	7%	924,1036,1066		16,24,h24	6%	924,1036		16,24
e. Hex-HexNAc-Hex-Hex-Cer	<5%	1155,1267,1297	450,654	16,24,h24			n.d.	
f. Hex-Hex-HexNAc-Hex-Hex-Cer	8%	1359,1471,1501	654,858,1062	16,h24			n.d.	
g. Fuc-Hex-HexNAc-Hex-Hex-Cer		1329,1441,1471	624,828,1032	16,24,h24?			n.d.	
h. Hex-HexNAc-Hex-HexNAc-Hex-Hex-Cer		1590,1702	450,654,858,1062	16,24			n.d.	
i. Hex-Hex-HexNAc-Hex-HexNAc-Hex-Hex-Cer	5%	1794,1936	654,858,1089	16,h24			n.d.	
j. Fuc-Hex-HexNAc-Hex-HexNAc-Hex-Hex-Cer		1764,1906	624,828,1059	16,h24			n.d.	
k. GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (globoside)			n.d.		5%	1155,1267	464	16,24
l. GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (Forssman antigen)			n.d.		21%	1386,1498	505,709	16,24
Others					<3%			
Acidic glycosphingolipids								
m. SO_4^{2-}-Gal-Cer (sulfatide)			n.d.				Present	
n. NeuAcα2-3Gal1-4GLc-Cer (hematoside)			Present				Present	

[a]Hex means hexose and HexNAc N-acetylhexosamine.
[b]Estimated amount is based on the composition of cells grown on filters and solubilized by SDS. The individual glycosphingolipids were quantitated by their sphingosine content as described in Materials and methods.
[c]Major F-fragments refer to fatty acid−carbohydrate ions found in the mass spectra of the permethylated-reduced samples.
[d]Sequence ions refer to ions in mass spectra containing different numbers of sugars from the non-reducing end. The underlined figures refer to the analysis of the permethylated sample.
[e]The fatty acid species listed each represent major fatty acids (over 20%) N-linked to the sphingosine base of the particular GSL. All major fatty acids were fully saturated, h means monohydroxylated fatty acids. The major sphingosine base in both cell lines was sphingosine (d18:1).
n.d., not detectable.

spectra) and are most likely lactosylceramide and globotriaosylceramide although specific antibodies are required to confirm this. The major GSL in the MDCK strain II cells (a and b, Table II) had only one sugar and was a mixture of glucosylceramide (<10%) and galactosylceramide (>90%) as revealed by t.l.c. on borate-impregnated plates (Karlsson *et al.*, 1973; not shown).

The MDCK strain I cells had GSLs with one, two and three sugars in common with the MDCK strain II cells (a, b, c and d, Table II) but the MDCK strain I had more than 90% of glucosylceramide which constituted less than 10% of the monoglycosylceramide in MDCK strain II. The larger GSLs of MDCK strain I were more complex than those of MDCK strain II. A series of peaks at *m/z* 1329−1529 in the spectra of the permethylated-reduced neutral GSLs from the MDCK strain I cells corresponded to at least two 5-sugar components (Figure 3A). The major one of these (f, Table II) had the probable sequence shown in the formula at the top of the mass spectra, Hex-Hex-HexNAc-Hex-Hex. This sequence was derived from sequence ions from the non-reducing end of the sugar chain found at *m/z* 219, 187 (219 minus 32), 654, 858 and 1062 of the permethylated-reduced sample and at *m/z* 219, 668, 636 (668 minus 32) and 872 of the permethylated sample (not shown). The possibility that this N-acetylhexosamine would be located at the fourth instead of the third position from the ceramide is less likely due to the relative intensities of the different carbohydrate sequence ion peaks. The second 5-sugar compound intermingled with these peaks had the terminal hexose replaced by a fucose (g, Table II). This gave peaks 30 mass units lower as compared with the major 5-sugar component. Diagnostic peaks were found at *m/z* 1329 (for the 16:0 non-hydroxy fatty acid), 189 (terminal fucose), 624, 828 and 1032 of the permethylated-reduced sample and at *m/z* 638, 606 (638 minus 32) and 842 of the permethylated sample. A probable precursor of the two 5-sugar compounds with four sugars and similar fatty acid distribution (e, Table II) was found in spectra recorded at lower temperatures. The series of peaks at *m/z* 1590 to 1702 correspond to a compound (h, Table II) having one N-acetylhexosamine and one hexose added to compound e. An addition of terminal fucose or hexose to this compound gave compounds j and i shown in the mass spectra of Figure 3A, and these GSLs were thus analogous to the e, g and f compounds. The 5- and 7-sugar GSLs corresponded to bands seen on the thin-layer plate of Figure 1. Mass spectra of the permethylated sample contained a large peak at *m/z* 182 indicating a 1−4 linkage to the internal HexNAc. The monoclonal anti-blood group H type 2 antibody should bind to the GSLs with 5- and 7-sugars (g and j, Table II) if the fucose in these com-

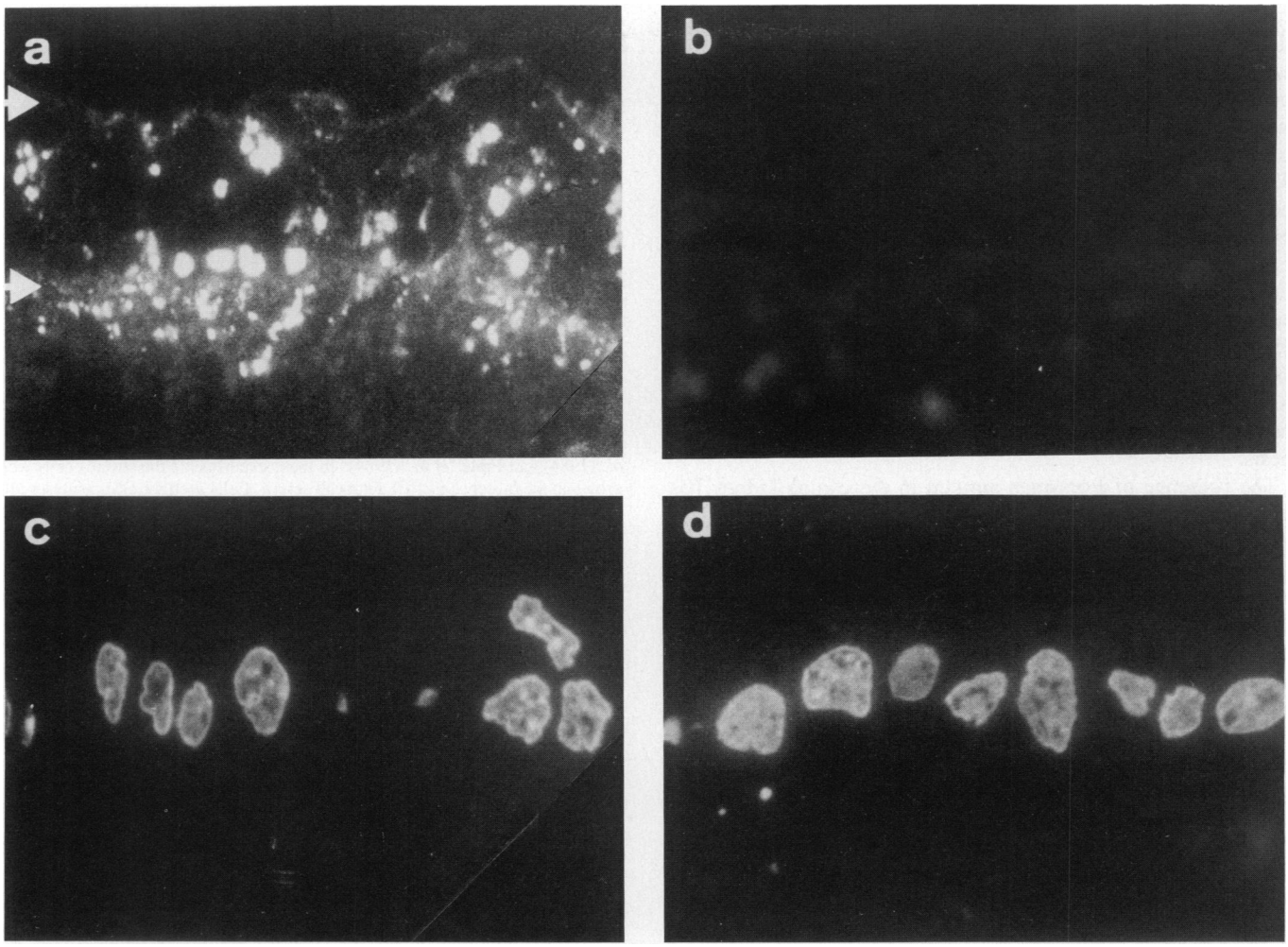

Fig. 4. Indirect immunofluorescence of MDCK strain II **(a)** and I **(b)** cells on nitrocellulose filters with anti-Forssman antibody. Semi-thin frozen sections were prepared and stained with antibodies as described in Materials and methods. The corresponding photographs **c** and **d** show the localization of the nuclei after Hoechst staining. The nitrocellulose filter is located at the lower side of the photographs. The arrows in panel **a** indicate the apical and basal sides of the cell monolayer. On the basal side the cells send projections into the filter. The cell monolayer in panels **a** and **c** is slightly curled on the right side of the photograph. Magnification is 1417×.

pounds were bound α 1−2 to neolactotetraosylceramide (a type 2 chain). Instead, this antibody bound to both MDCK strain I and II cells in the 6-sugar interval (Figure 2, lane C). The weak binding (when compared with the globoside in MDCK strain II) of the serum from the blood group p^k woman to the 5- and 7-sugar interval of MDCK strain I cannot be interpreted since this serum contains as yet uncharacterized antibodies in addition to the globoside antibodies. The possibility that the fucosylated 5-sugar compound would be the X-hapten (Yang and Hakomori, 1971) which has been shown to occur in dog intestine (McKibbin *et al.*, 1982) was excluded by the fact that it was not recognized by an anti-blood group X antibody. Also the possibility that this compound would be identical to fucosylated gangliotetraosylceramide is less likely as this reference migrated lower down on thin-layer plates (not shown). Most likely the 5- and 7-sugar compounds were GSLs that have not been described yet. Also, the (minor) GSL recognized by the anti-blood group H type 2 antibody in the 6-sugar interval in both cell strains remained unidentified. Obviously, more work is needed to elucidate these structures.

The major sphingosine base in both cell strains was sphingosine (d18:1). The one and two sugar components of MDCK strain II contained some hydroxy fatty acids with 16−24 carbons, whereas the more complex GSLs only contained non-hydroxy fatty acids (Table II). All GSLs of the MDCK strain I cells contained similar fatty acids as they had mostly non-hydroxy and hydroxy 16:0, 22:0 and 24:0 fatty acids. They also contained more than usual non-hydroxy and hydroxy 26:0 fatty acids.

The Forssman antigen was localized in filter-grown MDCK strain I and II cells by immunofluorescence. Frozen sections cut perpendicular to the monolayer were labeled with anti-Forssman antibody and stained with rhodamine-labeled anti-IgG (Figure 4). The apical surface of MDCK strain II cells was stained. No staining was seen on the basolateral surface. Intracellularly, vacuoles were heavily stained. The apical staining was confirmed by staining filter-grown MDCK strain II cells directly from the apical side and when anti-Forssman antibodies were applied to the cells from the basolateral side only, no staining was seen (not shown). In contrast, as expected from the chemical results, MDCK strain I cells were not labeled by the anti-Forssman antibody (Figure 4b).

Discussion

In the present study we demonstrate that the closely related MDCK strain I and II cells express the same proportion of different neutral and acidic GSLs but of different species. Both cell

strains originate from the cell line isolated from dog kidney by Madin and Darby in 1958 (Gaush *et al.*, 1966).

The differences in neutral GSLs (which account for 85% of the total GSLs) between the two strains can be reduced to the presence of two different series of GSLs. Although both cell strains express glucosylceramide, only MDCK strain II cells use this GSL as a precursor for globoside (globotetraosylceramide), for the Forssman antigen (globopentaosylceramide) and most likely also for the 2- and 3-glycosyl residue-containing members of this series which is commonly called the globo series (Makita and Taniguchi, 1985). MDCK strain I cells express GSLs of a different, not yet fully characterized, series containing also fucosyl-GSLs. These findings make the MDCK strain I and II cells valuable experimental systems for studying the biosynthesis of these two different GSL series and for characterizing the enzymes responsible for bifurcation into the two pathways of GSL synthesis.

The presence of Forssman antigen in the canine kidney has been chemically demonstrated by Sung *et al.* (1973). Interestingly, Tanaka and Leduc (1956) demonstrated by immunohistochemical techniques that the Forssman antigen is present in the dog kidney in epithelial cells of the ascending and convoluted segments of the distal tubule. Also, variable staining of the collecting ducts was observed. Thus the strain II cells could derive from segments positive for Forssman antigen and the strain I cells from negative segments. This interpretation would imply that the two MDCK strains represent different subpopulations of the cells present in the original isolate and that they derive from different epithelial cell types in the nephron. However, the possibility cannot be excluded that the different GSL patterns represent two differentiation stages of the same cell type. The globo series have been shown to be temporarily expressed during differentiation in embryos and certain cell cultures (Willison and Stern, 1978; Hakomori and Kannagi, 1983; Kannagi *et al.*, 1983).

MDCK strain II cells express sulfatide (galactosylceramide-3-sulfate) which is not detectable in MDCK strain I cells. Sulfatide has been identified in MDCK cells before by Ishizuka *et al.* (1978). Sulfatides were earlier proposed to be linked to ion transport because the Na^+-K^+-ATPase activity correlated with the quantity of sulfatide in several tissues (Karlsson *et al.*, 1974b; Hansson *et al.*, 1978). However, this correlation must be indirect since sulfatides have been shown to be localized on the apical surface of renal tubular cells (Zalc *et al.*, 1978) whereas the Na^+-K^+-ATPase is basolateral and therefore these cannot directly interact with each other (Karlsson, 1982).

Whether GSLs are asymmetrically distributed over the MDCK cell surface remains to be tested. The Forssman antigen at least appears by immunofluorescence microscopy to be preferentially localized in the apical membrane of MDCK II cells (Figure 4). A simple calculation shows that the GSLs cannot only be localized in the extracytoplasmic leaflet of the apical membrane (van Meer *et al.*, 1985). The mean surface areas of the MDCK strain I and II cells have been determined morphologically (von Bonsdorff *et al.*, 1985). The total apical surface area of a monolayer of MDCK strain I and II cells on a 20 mm diameter filter is 5.8 cm^2 and 9.6 cm^2, respectively. If the mean surface area of the GSLs in MDCK cells (Table II) is taken to be 48 $Å^2$ (Maggio *et al.*, 1978) and if one cholesterol molecule (35 $Å^2$, Levine and Wilkins, 1971) is allowed per GSL molecule in the plasma membrane, the outer leaflet of the apical membrane could contain maximally 1.2 nmol and 1.9 nmol of GSLs in MDCK strain I and II, respectively. Since a monolayer on a filter contains 4.7 nmol and 4.6 nmol of GSLs, 75% and 60% of the GSLs in MDCK strain I and II cells, respectively, have to be localized either on the basolateral membrane domain or intracellularly. Evidence for intracellular localization of part of the Forssman antigen in dog kidney has been published (Tanaka and Leduc, 1956), and our data confirm this in MDCK strain II cells. We are currently localizing the individual GSL species in MDCK cells more precisely by electron microscopy of thin frozen sections in an attempt to determine how specific the intracellular sorting of GSLs is in these cells.

Materials and methods

MDCK strain I and II cells

Both strain I and II cells used in our laboratory have been cloned, strain I for high resistance (Fuller *et al.*, 1984) and strain II for blister formation (Louvard, 1980). The chromosomes of both strains corresponded to the dog karyotype and both strains were found to be free of mycoplasma contamination by Hoechst staining. Nomenclature and growth conditions are discussed in Balcarova-Ständer *et al.* (1984) and Fuller *et al.* (1984). In these experiments the strain I cells were grown in the presence of 10% fetal calf serum. Cells used for GSL analyses (10^9 cells), were scraped from the plastic support as confluent monolayers 48 h after plating (no domes were present yet). The cells were centrifuged at 4°C for 5 min at 200 g_{max}, frozen and lyophilized.

Final quantitative determinations were carried out with cells grown on permeable supports since detailed morphological data have been reported for these systems (von Bonsdorff *et al.*, 1985). Cells were grown as described by Fuller *et al.* (1984) for strain I and by Matlin and Simons (1984) for strain II cells. Confluent monolayers of cells on 20 mm diameter nitrocellulose filters with a pore size of 0.45 μm were taken for analysis at 4 (strain I cells) or 3 days (strain II cells) after seeding. Cells on filters were counted *in situ* after staining of the nuclei with Hoechst dye as described (Fuller *et al.*, 1984). At the time of analysis the monolayers of MDCK strain I cells consisted of $2.6 \pm 0.5 \times 10^6$ cells/filter, the monolayers of strain II cells of $3.2 \pm 0.3 \times 10^6$ cells/filter. These numbers represent the mean followed by the standard deviation (n=19 and 15, respectively) of three independent filters for each cell type.

Purification of GSLs

For the GSL analysis of MDCK cells from a plastic support, 10^9 lyophilized cells were extracted in tubes with Teflon-lined screw caps, first with methanol and then three times with chloroform/methanol 1:2 (v/v), each time 30 min at 70°C followed by centrifugation at 900 *g* for 5 min. The combined supernatants were treated with 0.2 M KOH in methanol and dialyzed (Breimer *et al.*, 1981b). The nonpolar lipids were eluted on 1 g silicic acid by 10 ml of chloroform and 10 ml of 2% methanol in chloroform (v/v). The polar lipids were eluted with 10 ml of 75% methanol in chloroform (v/v) and 10 ml methanol (Karlsson, 1986) in one tube. The neutral GSLs were prepared from the combined fractions by DEAE-cellulose chromatography, acetylation and silicic acid chromatography as described (Breimer *et al.*, 1981b). The neutral GSLs were deacetylated overnight by 0.2 ml of a mixture of 2 ml 0.2 M KOH, 14 ml methanol and 5 ml toluene according to Karlsson (1986). The acid GSLs eluted by 5% LiCl from the DEAE-cellulose were dialyzed (Breimer *et al.*, 1981b) and applied to a 0.5 g silicic acid column, washed with 5 ml of 5% chloroform in methanol (v/v) and the GSLs were eluted with 75% and 100% methanol in chloroform.

In order to obtain the total GSLs from MDCK cells from nitrocellulose filters, 10 filters with MDCK strain I cells or MDCK strain II cells were extracted as a whole and the GSLs prepared as described above. As an alternative protocol for each cell strain, 30 filters were washed extensively with phosphate-buffered saline and incubated in 2% SDS in water at 80°C for 1 min. This was repeated once, which resulted in 25 ml SDS extracts. The extracts were lyophilized and the lyophilisate was extracted, treated with 0.2 M KOH, dialyzed and subjected to silicic acid chromatography as described above. The polar lipids from these fractions were acetylated by acetic acid anhydride/pyridine/chloroform 1:1:1 (v/v) and applied to a silicic acid column, where the GSLs were eluted by 5 and 10% methanol in chloroform (v/v). These fractions were deacetylated and subjected to DEAE-cellulose chromatography.

Analyses of GSLs

The GSL mixtures obtained were analysed by t.l.c. using HP-TLC Si50 (Merck, Darmstadt, FRG) plates. Solvent systems used are described in the figure legends. GSLs were detected by the anisaldehyde reagent (Stahl, 1962), the gangliosides by the resorcinol reagent (Svennerholm, 1963). Primuline (Skipski, 1975) was used for localization of the GSLs before a sphingosine assay. Detection of different antigens on GSLs was done by overlaying the thin-layer plate with antibodies (Magnani *et al.*, 1982; Hansson *et al.*, 1983). The following antibodies were used; monoclonal anti-blood group A, B and H type 2 from DAKO-patts (Copenhagen, Denmark), antibody 10 cl 17 reacting with Le^b and blood group H type

1 (Brockhaus *et al.*, 1981), and antibody $D_1$56-22 reacting with blood group X, which were kindly provided by Dr H.Koprowski, The Wistar Institute, Philadelphia, PA, and monoclonal anti-Forssman antibody obtained from culture supernatants of ATCC TIB 121 (American Type Culture Collection, Rockville, MD) (Willison and Stern, 1978). The serum containing anti-globoside antibodies was obtained by plasmapheresis of a pregnant woman of blood group p^k (Hansson *et al.*, in preparation). Antibodies against total mouse immunoglobulins and human IgG were obtained from DAKO-patts (Copenhagen, Denmark) and iodinated by the Iodogen method (Pierce, Rockford, IL). Kodak XAR-5 films were used for autoradiography.

The sphingosine content of the GSL mixtures or of individual GSLs separated by high performance t.l.c. was assayed by the method of Naoi *et al.* (1974) as performed by Bouhours and Glickman (1976).

One-fifth of the total neutral GSL mixtures were permethylated (Hakomori, 1964) and part of these further $LiAlH_4$-reduced (Karlsson, 1974). These samples were analysed by a VG-ZAB HF mass spectrometer (Manchester, UK) operating in the electron impact mode using the direct inlet system. The spectra were recorded at a trap current of 500 μA, an electron energy of 40 eV and accelerating voltage of 8 kV. The data were processed by a VG 11-250 data system. Paper spectra were counted by hand to give nominal masses reproduced.

Immunofluorescence

Frozen sections were cut from filter-grown MDCK cells and stained with the appropriate antibodies as described by Tokuyasu (1973). Filter-grown MDCK cells were stained for immunofluorescence from the apical or the basolateral sides as described by Fuller *et al.* (1984). The antibodies used were the monoclonal anti-Forssman antibody mentioned above and a rhodamine-labeled antibody against total mouse immunoglobulins.

Analyses of phospholipids and cholesterol

Phospholipids were extracted from cell monolayers on filters, separated by two-dimensional high performance t.l.c. and quantitated by micro-phosphor determination as described (van Meer and Simons, 1982). Cholesterol and cholesterolester were determined by an enzymatic assay (Sigma Chemical Co., St. Louis, MO) after separation by one-dimensional t.l.c. in a solvent system of hexane/diethylether/acetic acid (90:10:1 v/v).

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