Neutral Glycosphingolipids and Gangliosides of Human Lung and Lung Tumours

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In order to help determine whether alterations of the profiles of glycosphingolipids occur consistently in human tumours, the neutral glycosphingolipids and gangliosides of nine lung tumours (one adenocarcinoma, four squamous cell, two mixed adeno-squamous cell, one large cell and one oat-cell carcinomata) were analysed. The control tissue consisted of adjacent lung; it contained neutral glycosphingolipids corresponding in properties to glucosyl-, lactosyl-, globotriaosyl- and globotetraosyl-ceramides. All of the tumours also contained these four neutral glycosphingolipids. However, in addition, five of the tumours (two of the squamous, the large cell and the two mixed adeno-squamous cell carcinomata) contained neutral glycosphingolipids corresponding in properties to lactotriaosyl- and neolactotetraosyl-ceramides; these same tumours also exhibited higher amounts of lactosylceramide than the other tumours analysed. Both of the two former neutral glycosphingolipids and very substantial amounts of the latter neutral glycosphingolipid were detected in pneumonic lung and in polymorphonuclear leucocytes; it thus appears possible that these particular compounds were derived from these latter cells, rather than from the tumour cells. The ganglioside patterns of the tumours were almost equivalent in complexity to that exhibited by the control lung tissue. This study shows that the profiles of two major classes of glycosphingolipids (neutral glycosphingolipids and gangliosides) occurring in lung tumours are almost as complex as those of the parent tissue, a finding in contrast with the notably simplified patterns of these lipids found in many cancer cells grown *in vitro*. It also suggests that when lactotriaosyl- and neolactotetraosyl-ceramides and high amounts of lactosylceramide are detected in human tumours, the possibility must be considered that these compounds are derived from polymorphonuclear leucocytes.

A number of investigations have demonstrated notable simplifications of the pattern of gangliosides in virally transformed cells grown *in vitro* [reviewed by Brady & Fishman (1974), Hakomori (1975a) and Sweeley & Siddiqui (1977)]. Simplifications of the ganglioside patterns of chemically transformed rat hepatocytes growing *in vitro* (Brady *et al.*, 1969) and *in vivo* (Siddiqui & Hakomori, 1970; Cheema *et al.*, 1970) have also been detected; the alterations in

Abbreviations used: GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; AcNeu, *N*-acetylneuraminic acid; GcNeu, *N*-glycolylneuraminic acid; Cer, ceramide (2-*N*-acylsphingosine); GL-1, glucosylceramide [Glc β 1 $\rightarrow$ 1' Cer]; GL-3, globotriaosylceramide [Gal(α 1-4)Gal(β 1-4)Glc β 1 $\rightarrow$ 1' Cer]; GL-4, globotetraosylceramide [GalNAc(β 1-3)Gal(α 1-4)Gal(β 1-4)Glc β 1 $\rightarrow$ 1' Cer]; lactotriaosylceramide, GlcNAc(β 1-3)Gal(β 1-4)Glc β 1 $\rightarrow$ 1' Cer; neolactotetraosylceramide, paragloboside, Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc β 1 $\rightarrow$ 1' Cer. The nomenclature for the gangliosides is that of Svennerholm (1964).

ganglioside patterns were less profound in hepatoma cells growing *in vivo* and involved primarily the most complex gangliosides (i.e. di- and tri-sialo species). To determine whether changes in glycosphingolipid composition occur in human tumour cells growing *in vivo*, several workers have examined the profiles of these components present in certain tumours. Karlsson *et al.* (1974) studied the sphingolipid composition of several renal carcinomata. The ganglioside patterns and total neuraminic acid contents of human gastric and colon carcinomata were reported by Keränen *et al.* (1976). Siddiqui *et al.* (1978) described the results of extensive studies on the glycosphingolipids of colonic adenocarcinomata. Although some of the tumours analysed in these studies did show alterations in glycosphingolipid composition in comparison with the control tissues (for further details see the Discussion), in general the changes detected were much less than those noted in many cultured transformed cells.

In the present work we have compared the neutral glycosphingolipid and ganglioside contents of a number of human lung tumours with those of adjacent uninvolved lung tissue. The results have revealed that

the profiles of these two major classes of glycosphingolipids in the tumours were almost as complex as those of lung. In addition, evidence is presented that suggests that when lactotriaosyl- and neolactotetraosyl-ceramides and elevated amounts of lactosylceramide are detected in human tumours, the possibility must be considered that these components are derived from contaminating polymorphonuclear leucocytes.

Materials and Methods

Collection of lung tissue and lung tumours

The tumours analysed were obtained through the co-operation of colleagues in the Department of Surgical Pathology, Toronto General Hospital, Toronto, Ont., Canada. All specimens were collected within several hours of removal. In each case, adjacent uninvolved lung tissue was also obtained that had been removed during surgical resection of the tumours. One specimen of pneumonic lung (lobar pneumonia) was also obtained from the autopsy room of the same hospital. Before lipid extraction, all the tumours were washed in ice-cold 0.9% NaCl, and any macroscopically visible areas of necrosis or infection were removed as carefully as possible.

Isolation of neutral glycosphingolipid and ganglioside fractions

Extraction of lipids was performed by the method of Suzuki (1965). The upper and lower phases of the extracts obtained by this procedure were subjected to methanolysis in mild alkali as described previously (Narasimhan *et al.*, 1976); preliminary experiments revealed that none of the glycolipids of the lung and tumours studied were affected by this treatment. The lipids of the lower and upper phases were then fractionated by silicic acid column chromatography (Vance & Sweeley, 1967; Yogeeswaran *et al.*, 1972). Lipid-bound sialic acid in the ganglioside fraction was estimated by the resorcinol method of Svennerholm (1957) as modified by Miettinen & Takki-Luukkainen (1959).

Separation and analysis of gangliosides and neutral glycosphingolipids by t.l.c.

Samples of the neutral glycosphingolipid and ganglioside fractions were subjected to t.l.c. on glass plates coated with silica gel G (250 μ m thickness; Merck A.G., Darmstadt, West Germany) as described previously (Narasimhan *et al.*, 1976); the standard solvent systems were chloroform/methanol/conc. NH_3 /water (65:35:1:7, by vol.) for gangliosides and chloroform/methanol/water (65:25:4, by vol.) for neutral glycosphingolipids. Aniline/diphenylamine reagent (Smith, 1960) was used to detect neutral

glycosphingolipids, and resorcinol reagent (Wherrett & Cumings, 1963) to detect gangliosides. Quantitative analyses of the distribution of sialic acid in the major gangliosides of the lung and certain of the tumours were performed by the method of MacMillan & Wherrett (1969). For the purposes of structural studies, gangliosides and neutral glycosphingolipids were separated by preparative t.l.c., their locations detected by brief exposure of guide-strips to iodine vapour, the appropriate areas of the chromatograms marked and then taken for further analyses.

Identification of the sialic acid and neutral glycosphingolipid components of certain gangliosides by partial hydrolysis in mild acid

This was performed as described previously (Yogeeswaran *et al.*, 1972).

Analyses by g.l.c. of carbohydrates, fatty acids and long-chain bases

The sugar, fatty acid and long-chain base compositions of purified individual neutral glycosphingolipids obtained from extracts of normal lung and from certain of the tumours were analysed by g.l.c. All analyses by this method were performed by using a Hewlett-Packard (model 5830-A) instrument, equipped with dual glass columns (234 mm $\times$ 2 mm internal diameter) and flame-ionization detectors. The liquid support was 3% (w/w) OV-1 on acid-washed Chromosorb P (80–100 mesh), with helium as the carrier gas. Sugars were analysed as the trimethylsilyl ethers of their *O*-methyl glycosides (Vance & Sweeley, 1967) after acetylation of amino sugars (Clamp *et al.*, 1967; Yogeeswaran *et al.*, 1973). Fatty acids were analysed as their methyl esters on the same columns (Yogeeswaran *et al.*, 1972); to determine if hydroxy fatty acids were present, appropriate portions of the methyl esters of the fatty acids of certain glycolipids were subjected to trimethylsilylation and then re-analysed under the same conditions. Analyses of long-chain bases by g.l.c. were performed by using the trimethylsilyl derivatives of the acetylated compounds (Carter & Gaver, 1967; Singh, 1973).

Analyses of the long-chain bases of certain neutral glycosphingolipids by g.l.c.–mass spectrometry

These analyses were made by coupling a Varian MAT CH-5 single focusing mass spectrometer to the Varian model 2700 gas chromatograph (equipped with 1% SE-30 columns) by means of a Watson-Biemann molecular separator (Marai *et al.*, 1976). The mass spectrometer was in turn coupled to a Varian 6201 computer. These analyses were performed by Mr. L. Marai and Dr. A. Kuksis, Banting and Best Department of Medical Research, University of Toronto.

Separation by t.l.c. of the acetylated species of globotriaosylceramide and lactotriaosylceramide

Portions of a mixture of these two compounds obtained by preparative t.l.c. of the neutral glycosphingolipid extracts of normal lung and of the lung tumours were taken to dryness and then acetylated by the method of Saito & Hakomori (1971). The acetylated glycolipids were also taken to dryness, dissolved in chloroform/methanol (2:1, v/v) and then subjected to t.l.c. in a solvent system of 1,2-dichloroethane/methanol (9:1, v/v) (Joseph & Gockerman, 1975).

Preparation of human polymorphonuclear leucocytes

Three different preparations of polymorphonuclear leucocytes were prepared from freshly collected human blood (approx. 100ml each) by the method of Böyum (1968). The cells were over 95% pure, as evaluated by microscopic examination of smears stained with Wright's stain (Methylene Blue/Eosin) (Williams, 1972). Approx. 10^8 cells were obtained in each preparation. The preparations of these cells were performed by Ms. R. Rachkewich and Dr. K. Shumak of the Department of Medicine, University of Toronto.

Immunodiffusion

The identity of the putative neolactotetraosylceramide species isolated from certain of the lung tumours was investigated by using an antiserum (raised in a rabbit) to neolactotetraosylceramide. The antiserum was supplied by Dr. S. Hakomori, University of Washington, Seattle, Washington (cf. Siddiqui & Hakomori, 1973). Analyses by immunodiffusion were performed by using 1% (w/v) Ionagar no. 2 slabs (Oxoid Ltd., London E.C.4, U.K.) in Petri dishes. The centre wells were filled with the antiserum and the outer wells with alternating samples of the putative neolactotetraosylceramide species from certain of the tumours and with samples of the authentic glycolipid, all suspended in 0.9% NaCl. Results were observed from 18 to 36h after commencement of diffusion.

Standards of glycosphingolipids

Gangliosides and neutral-glycosphingolipid standards were prepared from human brain and kidney by the same procedures as described for the extraction and isolation of these compounds from the lung and lung tumours. A standard of neolactotetraosylceramide (prepared from human polymorphonuclear leucocytes) (Wherrett, 1973) was generously provided by Dr. J. R. Wherrett, Department of Medicine, University of Toronto. A standard of lactotriaosyl-

ceramide (prepared by degradation of a pentaglycosylceramide from rabbit bone marrow) was generously provided by Dr. S. Basu, Notre Dame University, South Bend, IN, U.S.A.

Protein analyses

The concentrations of the glycolipids analysed were related to the protein contents of the non-lipid residues obtained during the initial lipid extraction. Protein estimations were performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Fig. 1 shows the results of analyses by t.l.c. of the neutral glycosphingolipids of the control tissue and of the various lung tumours. Channel 4 (Fig. 1a) shows the neutral glycosphingolipids of one sample of the former tissue; compounds (all resolved as double bands) corresponding in migration to standards of glucosyl-, lactosyl-, globotriaosyl- and globotetraosyl-ceramides are evident. The chromatographic migrations of galactosylceramide and of the latter compounds are shown in channel 5. The patterns exhibited by five other specimens of control lung tissue were very similar to that shown in channel 4. Channels 1 and 2 show the neutral glycosphingolipids of two of the lung tumours (squamous-cell tumours A and B respectively); the patterns are quite similar to each other and also generally resemble that of the control tissue. The neutral-glycosphingolipid profile of a specimen of lung heavily infiltrated by polymorphonuclear leucocytes is also shown for comparison in channel 3; however, comments on this pattern are deferred until Fig. 2 is described. Fig. 1(b) shows the neutral glycosphingolipids of the other lung tumours studied. The migrations of relevant standards are shown in channels 1 and 9. The patterns of squamous-cell tumours C and D (channels 2 and 3) are generally similar to each other, although the former shows little or no galactosylceramide. Compounds corresponding in migration to lactosyl-, globotriaosyl- and globotetraosyl-ceramides are prominent in both of these tumour extracts; in addition, both tumours exhibit a compound (more prominent in the case of D) corresponding in migration to neolactotetraosylceramide (cf. channel 1). Of particular significance is the fact that the bands corresponding in migration to lactosylceramide are relatively much more prominent in the case of these two squamous-cell tumours than in that of the two similar types of tumour shown in Fig. 1(a) (channels 1 and 2). The neutral glycosphingolipids of the sole adenocarcinoma studied are shown in channel 4; compounds corresponding in migration to glucosyl-, galactosyl-, lactosyl-, globotriaosyl- and globo-

Fig. 1. *T.l.c. of the neutral glycosphingolipids of various lung tumours and of normal lung*

(a) Thin-layer chromatogram of the neutral glycosphingolipids of: 1, squamous-cell tumour A; 2, squamous-cell tumour B; 3, lung infiltrated by polymorphonuclear leucocytes (pneumonic lung); 4, normal lung. Channel 5 contained a mixture of standard neutral glycosphingolipids. (b) Thin-layer chromatogram of neutral glycosphingolipids: 1, standard of neolactotetraosylceramide; then, neutral glycosphingolipids of: 2, squamous-cell tumour C; 3, squamous-cell tumour D; 4, adenocarcinoma; 5, large-cell carcinoma; 6, oat-cell carcinoma; 7, mixed adeno-squamous carcinoma A; 8, mixed adeno-squamous carcinoma B. Channel 9 contained a mixture of standard neutral glycosphingolipids. The chromatograms were developed in chloroform/methanol/water (65:25:4, by vol.) and sprayed with the aniline/diphenylamine reagent. The compounds indicated by the white dots were not neutral glycosphingolipids. The standard neutral glycosphingolipids in channels 5 and 9 of (a) and (b) respectively were (from top to bottom) galactosyl-, lactosyl-, globotriaosyl- and globotetraosyl-ceramides. The labels in the right-hand margin indicate the chromatographic positions of the following neutral glycosphingolipids: GL-1, mixture of glucosyl- and galactosyl-ceramides; GL-2, lactosylceramide; GL-3, mixture of globotriaosyl- and lactotriaosyl-ceramides; GL-4, globotetraosylceramide. Patterns essentially identical with those illustrated were observed after similar analyses by t.l.c. of two other portions of the neutral-glycosphingolipid fraction of each sample indicated above.

tetraosyl-ceramides are seen. Two features of additional interest are that the relative amount of lactosylceramide is considerably lower than seen in the extracts of squamous-cell tumours C and D and that only a trace of a compound corresponding in migration to neolactotetraosylceramide is evident. The profile of the single large-cell carcinoma studied is shown in channel 5; the pattern resembles that of squamous-cell tumour C, and, in particular, compounds corresponding in migration to lactosyl- and neolactotetraosyl-ceramides are prominent. In contrast, the single oat-cell carcinoma studied (channel 6) exhibits a pattern similar to that of the adenocarcinoma (channel 4), with a relatively low amount of lactosylceramide and no neolactotetraosylceramide. The patterns of the two mixed adeno-squamous-cell carcinomata (channels 7 and 8) are generally similar to each other; of especial interest is that both show relatively high amounts of lactosylceramide and also moderate amounts of the compound corresponding in migration to neolactotetraosylceramide.

As shown in Fig. 1, the tumours studied showed considerable variations in their relative contents of

lactosyl- and neolactotetraosyl-ceramides. Although these variations could have represented true differences in the glycosphingolipid profiles of the various tumours, it appeared prudent to consider the possibility that they might also arise from the presence of a non-tumour cell type contaminating these tumours. As variable areas of infection (microscopic and/or macroscopic) secondary to bronchial obstruction are not uncommon in lung tumours (cf. Millard, 1977), the possibility that polymorphonuclear leucocytes might be contributing these components to the glycosphingolipid profiles of certain of the tumours was considered. The fact that the standard of neolactotetraosylceramide used in the present investigation was obtained from human polymorphonuclear leucocytes also lent a measure of support to this idea. Accordingly, the above possibility was examined by two approaches: firstly, by examining the glycosphingolipid profiles of several preparations of purified human polymorphonuclear leucocytes, and, secondly, by examining the glycosphingolipid profile exhibited by a specimen of pneumonic lung containing a heavy infiltration of these cells. Results of analysis by

t.l.c. of the neutral glycosphingolipids of the relevant cells and tissues analysed are given in Fig. 2. Channel 1 shows the chromatographic migration of a standard of neolactotetraosylceramide and channel 6 shows the migrations of the other principal standards. The neutral glycosphingolipids of a preparation of polymorphonuclear leucocytes are shown in channel 2; the most important features are the presence of very substantial amounts of compounds corresponding in migration to lactosyl- and neolactotetraosylceramides. The neutral-glycosphingolipid profiles of portions of the pneumonic lung and of control lung are shown in channels 3 and 4 respectively; the major differences between the two tissues are the presence in the former of substantial amounts of compounds corresponding in migration to lactosyl- and neolactotetraosylceramides (also cf. channels 3 and 4 of Fig. 1a). For comparison, the neutral-glycosphingo-

lipid profile of squamous-cell tumour D, one of the tumours exhibiting elevated amounts of lactosylceramide and the presence of neolactotetraosylceramide, is shown in channel 5.

The results of quantitative estimations by g.l.c. of the amounts of the principal neutral glycosphingolipids present in lung, in the tumours and in polymorphonuclear leucocytes are given in Table 1. Globotetraosylceramide (GL-4) was the major component of the normal lung. With regard to the results obtained by analyses of the various tumours, one feature is of particular interest, i.e. these tumours (squamous-cell carcinoma D, the two adenosquamous carcinomata and the large-cell carcinoma) that contained relatively large amounts of lactosylceramide (GL-2) also contained appreciable amounts of neolactotetraosylceramide. This finding confirmed the qualitative correlation between the amounts of these lipids made by t.l.c. (cf. Fig. 1). In addition, the results of the analyses of the polymorphonuclear leucocytes demonstrated that these cells contained the highest amounts of lactosyl- and neolactotetraosylceramides of the tissues studied.

Analyses by g.l.c. also yielded information on the carbohydrate compositions of the principal neutral glycosphingolipids of lung, the tumours and polymorphonuclear leucocytes (see Table 2). On the basis of such analyses, partial structures for the principal neutral glycosphingolipids of lung are presented in Table 2. The carbohydrate compositions of the major neutral glycosphingolipids of all of the tumours were investigated by g.l.c.; representative data for only two of the tumours are presented. The data for squamous-cell carcinoma A (which contained very little neolactotetraosylceramide) suggest that the major glycosphingolipids of this tumour were glucosyl-, galactosyl-, lactosyl-, globotriaosyl- and globotetraosylceramides. Essentially similar results were obtained from the analyses of squamous-cell carcinoma B, the adenocarcinoma and the oat-cell carcinoma (results not shown). The results of the analyses of squamous-cell carcinoma D revealed that it also contained the five neutral glycosphingolipids detected in squamous-cell carcinoma A. However, in addition it was found to contain a neutral glycosphingolipid (PG) whose carbohydrate composition was consistent with its being neolactotetraosylceramide. Another important feature was that the results from analysis of the GL-3 glycosphingolipid revealed the presence of appreciable amounts of GlcNAc; this observation was investigated further (see below). The analyses of the other four tumours (squamous-cell carcinoma C, the two adenosquamous carcinomata and the large-cell carcinoma) yielded results similar to those presented for squamous-cell carcinoma D. As shown in Table 2, results from the analyses of the neutral glycosphingolipids of the polymorphonuclear leucocytes indicated

Fig. 2. Thin-layer chromatogram of the neutral glycosphingolipids from various cells or tissues

1, Standard neolactotetraosylceramide; 2, polymorphonuclear leucocytes; 3, lung infiltrated by polymorphonuclear leucocytes (pneumonic lung); 4, normal lung; 5, squamous-cell tumour D; 6, standard neutral glycosphingolipids. The chromatogram was developed and sprayed as described in the legend to Fig. 1. The standard neutral glycosphingolipids and the significance of the areas of the chromatogram indicated by GL-1, GL-2, GL-3 and GL-4 are also described in the legend to Fig. 1. Compounds indicated by white dots were not neutral glycosphingolipids. Patterns essentially identical with those illustrated were observed after similar analyses by t.l.c. of two other portions of the neutral glycosphingolipid fraction of each sample indicated above.

Table 1. *Amounts of neutral glycosphingolipids in normal lung, the lung tumours and in polymorphonuclear leucocytes*
The neutral glycosphingolipids of each tissue were separated by preparative t.l.c. and analyses by g.l.c. were performed as described in the Materials and Methods section. Protein was measured by the method of Lowry *et al.* (1951). The amounts of the individual glycosphingolipids are expressed as nmol of glucose/mg of protein, except for galactosylceramide, which are expressed as nmol of galactose/mg of protein. The results are means of duplicate determinations on the neutral glycosphingolipid extracts of each tumour and of one preparation of polymorphonuclear leucocytes, except for lung, where the results represent the means of duplicate determinations on three different specimens. The variation between the results of the duplicate determinations was less than 10% for all of the samples analysed. Abbreviations: Tr., trace; N.D., not determined; PG (paragloboside), neolactotetraosylceramide.

Tissue	GL-1		GL-2	GL-3	GL-4	PG
	Glucosylceramide	Galactosylceramide				
Lung	0.16	0.02	0.30	0.29	0.61	—
Squamous-cell tumour A	0.84	0.58	1.02	0.03	0.02	—
Squamous-cell tumour B	0.36	0.05	0.44	0.05	0.06	—
Squamous-cell tumour C	0.15	0.03	1.30	0.46	0.27	Tr.
Squamous-cell tumour D	0.52	0.45	4.88	0.19	0.06	0.25
Adeno-squamous-cell carcinoma A	2.40	1.08	2.37	0.12	0.05	0.05
Adeno-squamous-cell carcinoma B	0.87	0.12	3.21	0.40	0.24	0.05
Adenocarcinoma	0.91	0.86	0.73	0.11	0.17	—
Large-cell carcinoma	0.22	0.06	8.14	0.13	0.09	0.22
Oat-cell carcinoma	1.95	1.30	0.71	0.33	0.26	—
Polymorphonuclear leucocytes	0.20	—	21.02	N.D.	N.D.	0.43

Table 2. *Results of analysis by g.l.c. of the carbohydrates of the neutral glycosphingolipids of normal lung, certain lung tumours and of polymorphonuclear leucocytes and also of the G_{M3} species of the two former tissues*

The various glycosphingolipids were isolated by preparative t.l.c. and their carbohydrate compositions analysed as described in the Materials and Methods section. Each analysis was performed in duplicate; the variation between the results of the duplicate determinations was less than 5% for all of the samples analysed. Abbreviations: Tr., trace; PG (paragloboside), neolactotetraosylceramide.

Tissue	Glycosphingo-lipids	Glc	Gal	GalNAc	GlcNAc	AcNeu	Tentative partial structure
Lung	GL-1	1.0	0.03	—	—	—	Glc-Cer and Gal-Cer (Tr.)
	GL-2	1.0	0.93	—	—	—	Gal-Glc-Cer
	GL-3	1.0	1.82	—	—	—	Gal-Gal-Glc-Cer
	GL-4	1.0	1.79	0.78	—	—	GalNAc-Gal-Gal-Glc-Cer
	G _{M3}	1.0	1.04	—	—	0.88	AcNeu-Gal-Glc-Cer
Squamous-cell tumour A	GL-1	1.0	0.99	—	—	—	Glc-Cer and Gal-Cer
	GL-2	1.0	1.14	—	—	—	Gal-Glc-Cer
	GL-3	1.0	2.07	—	—	—	Gal-Gal-Glc-Cer
	GL-4	1.0	1.98	0.89	—	—	GalNAc-Gal-Gal-Glc-Cer
	G _{M3}	1.0	0.99	—	—	0.70	AcNeu-Gal-Glc-Cer
Squamous-cell tumour D	GL-1	1.0	0.87	—	—	—	Glc-Cer and Gal-Cer
	GL-2	1.0	1.05	—	—	—	Gal-Glc-Cer
	GL-3	1.0	1.86	—	0.43	—	Gal-Gal-Glc-Cer and GlcNAc-Gal-Glc-Cer
	GL-4	1.0	1.87	0.70	—	—	GalNAc-Gal-Gal-Glc-Cer
	PG	1.0	1.75	—	0.80	—	Gal-GlcNAc-Gal-Glc-Cer
	G _{M3}	1.0	0.97	—	—	0.86	AcNeu-Gal-Glc-Cer
Polymorphonuclear leucocytes	GL-1	1.0	—	—	—	—	Glc-Cer
	GL-2	1.0	1.06	—	—	—	Gal-Glc-Cer
	PG	1.0	1.82	—	0.84	—	Gal-GlcNAc-Gal-Glc-Cer

that the principal compounds were glucosyl-, lactosyl- and neolactotetraosyl-ceramides.

Several other observations were made relating to the chemical compositions of the principal neutral glycosphingolipids of normal lung and the tumours. Analyses by g.l.c. of the fatty acid compositions of the four major neutral glycosphingolipids detected in lung, and of the corresponding compounds present in each of the tumours, revealed that they all contained a variety of fatty acids ranging in chain length from 14 to 24 carbon atoms. No consistent differences between the compositions of the lung and tumour glycosphingolipids were evident. In addition, no hydroxy fatty acids were detected in these glycolipids.

The long-chain bases derived from the mono-hexosyl-, lactosyl-, globotriaosyl- and globotetraosyl-ceramide species of three specimens of control lung, the oat-cell carcinoma and the adenocarcinoma were analysed by g.l.c. These analyses revealed that sphingosine (80–85%) and dihydrosphingosine (15–20%) were the principal long-chain bases. Analyses by g.l.c.–mass spectrometry of the same neutral glycosphingolipids (excluding globotetraosyl-ceramide) from squamous-cell tumour A confirmed the presence of these two long-chain bases. The mass spectra of sphingosine showed 'molecular-weight' ions at m/e 470 ($M-15$), 426 ($M-59$) and 311 ($M-174$), and of dihydrosphingosine at m/e 472 ($M-15$), 313 ($M-174$) and 217 ($M-270$).

The identities of the putative neolactotetraosyl-ceramide species isolated from squamous-cell carcinoma D, adeno-squamous-cell carcinoma B and the large-cell carcinoma were examined by using a specific anti-(neolactotetraosylceramide) serum. The compounds isolated from each of these tumours showed a line of immunoprecipitation with the anti-serum and a reaction of complete identity with the authentic compound.

The results of analyses by g.l.c. (Table 2) indicated the presence of GlcNAc in the GL-3 areas of chromatograms (see Fig. 1) of the neutral glycosphingolipid fractions of certain of the tumours (in fact, those tumours that displayed elevated amounts of lactosylceramide and the presence of neolactotetraosylceramide). This raised the possibility that this area of the chromatograms contained two distinct neutral glycosphingolipids, e.g. globotriaosyl- and lactotriaosyl-ceramides (the latter containing GlcNAc). As the acetylated species of these two lipids can be separated by t.l.c. (Joseph & Gockerman, 1975), the glycolipids of the GL-3 areas from extracts of pneumonic lung, control lung and of the tumours studied were isolated by preparative t.l.c. and subjected to acetylation. The results of analyses of these species by t.l.c. are shown in Fig. 3. The migration of acetylated standards of lactotriaosyl- and globotriaosyl-ceramides are shown in channel 1 of Figs. 3(a)

and 3(b) respectively. Pneumonic lung (channel 2, Fig. 3a) shows the presence of the acetylated species of both these compounds, whereas control lung (channel 3, Fig. 3a) exhibits mainly the acetylated species of globotriaosylceramide. The results of a similar analysis of the GL-3 area of squamous-cell tumour A (a tumour exhibiting little neolactotetraosylceramide) are shown in channel 4 of Fig. 3(a). The acetylated species of globotriaosylceramide is seen to be the principal component, although a small amount of lactotriaosylceramide is also present. The results of similar analyses of the GL-3 areas of squamous-cell tumour D, the large-cell tumour and of mixed adeno-squamous-cell tumours A and B (all of which tumours contained appreciable amounts of neolactotetraosylceramide) are shown in channels 2–5 respectively of Fig. 3(b). All of these tumours are seen to exhibit the acetylated species of globotriaosylceramide and also quite substantial amounts of the acetylated species of lactotriaosylceramide.

The ganglioside patterns of all the tumours studied were analysed by t.l.c. Results from these experiments are shown in Figs. 4(a) and 4(b). The patterns of standard gangliosides prepared from human brain are shown in channel 1 of each of the Figures. The ganglioside pattern of a preparation of control lung is shown in channel 2 of Fig. 4(a); compounds corresponding in migration to gangliosides G_{M3} , G_{M2} , G_{M1} and G_T are the principal components visible. The ganglioside patterns of squamous-cell tumours A and B (the two tumours of this type exhibiting little or no neolactotetraosylceramide, and thus, on the basis of this criterion, free of appreciable contamination by polymorphonuclear leucocytes) are shown in channels 3 and 4 of Fig. 4(a); both tumour extracts show various amounts of compounds corresponding in migration to gangliosides G_{M3} , G_{M2} and G_{M1} and a marked decrease in or absence of the zone corresponding to ganglioside G_T seen in the control lung tissue. The ganglioside patterns of the seven other tumours are shown in channels 2–8 of Fig. 4(b). All of these tumours also exhibit relatively complex patterns of gangliosides, with ganglioside G_{M3} being their principal component. The complex ganglioside patterns of the adenocarcinoma (channel 4) and the oat-cell carcinoma (channel 6) are of particular interest, in that these tumours (like squamous-cell tumours A and B) possessed little neolactotetraosylceramide (see Fig. 1). As an index of the complexity of these ganglioside profiles, both are seen to exhibit a band corresponding in migration to ganglioside G_T [the prominent G_T band in the analysis shown in channel 6 migrated just behind the standard of G_T ; however, a repeat analysis by t.l.c. (results not shown) demonstrated that this band did correspond in migration to G_T].

Quantification of the contribution of the major gangliosides to the total patterns resolved by t.l.c.

Fig. 3. Thin-layer chromatogram of the acetylated lactotriaosylceramide and globotriaosylceramide species of various tissues (a) Thin-layer chromatogram of: 1, standard of lactotriaosylceramide; 2, pneumonic lung; 3, normal lung; 4, squamous-cell tumour A. (b) Thin-layer chromatogram of: 1, human kidney; 2, squamous-cell tumour D; 3, large-cell tumour; 4, mixed adeno-squamous-cell tumour B; 5, mixed adeno-squamous cell tumour A. The chromatographic positions of the acetylated species of globotriaosylceramide and lactotriaosylceramide are indicated by X and Y respectively on the left-hand margin of (a). The neutral-glycosphingolipid extracts of the various tissues indicated above were subjected to preparative t.l.c., and the areas of the chromatograms corresponding to GL-3 (cf. Fig. 1) were scraped off and eluted. The glycolipids were then acetylated, subjected to t.l.c. in a solvent system of 1,2-dichloroethane/methanol (9:1, v/v), and the chromatogram was sprayed with the aniline/diphenylamine reagent. Patterns essentially identical with those illustrated were observed after similar analyses by t.l.c. of duplicate samples from each tissue and tumour indicated above.

was performed by the technique of MacMillan & Wherrett (1969). The results of the analyses are shown in Table 3. In general, these results confirmed that the compound corresponding in chromatographic migration to ganglioside G_{M3} was the principal ganglioside of both lung and the tumours.

The putative ganglioside G_{M3} species from lung (three specimens) and each of the tumours were isolated by preparative t.l.c. and subjected to partial characterization. The results of analyses of the carbohydrate compositions of the species isolated from lung and two of the tumours are presented in Table 2. These results indicated that the sugar compositions of these species were consistent with their being ganglioside G_{M3} . The results of analyses by g.l.c. of the species corresponding in migration to ganglioside G_{M3} obtained from the seven other tumours indicated that their carbohydrate compositions were also consistent with their being ganglioside G_{M3} . In addition,

use of the method of Yogeeswaran *et al.* (1972) on each of these species (i.e. from both normal lung and the tumours) revealed that mild acid hydrolysis liberated only *N*-acetylneuraminic acid and a neutral glycosphingolipid corresponding in migration to lactosylceramide. Only limited studies were performed on the more complex gangliosides of lung and certain of the tumours. Mild acid hydrolysis of the gangliosides corresponding in migration to G_{M1} and G_{D1a} present in normal lung liberated *N*-acetylneuraminic acid and a neutral glycosphingolipid corresponding in migration to a tetraglycosylceramide species. Similar results were obtained from analyses of the corresponding gangliosides purified from squamous-cell tumours C and D and the two adeno-squamous carcinomata. No *N*-glycoloylneuraminic acid was detected in any of the gangliosides analysed. Analyses of these latter gangliosides derived from these tumours indicated the presence of appreciable

Fig. 4. *T.l.c. of the gangliosides of normal lung and of various lung tumours*
(a) Thin-layer chromatogram of the gangliosides of: 1, human brain; 2, normal lung; 3, squamous-cell carcinoma A; 4, squamous-cell carcinoma B. (b) Thin-layer chromatogram of the gangliosides of: 1, human brain; 2, squamous-cell carcinoma C; 3, squamous-cell carcinoma D; 4, adenocarcinoma; 5, large-cell carcinoma; 6, oat-cell carcinoma; 7, mixed adeno-squamous-cell carcinoma A; 8, mixed adeno-squamous-cell carcinoma B. The chromatograms were developed in chloroform/methanol/conc. NH₃/water (65:35:1:7, by vol.) and sprayed with the resorcinol reagent. The chromatographic positions of the gangliosides G_{M3}, G_{M1} and G_T are indicated on the left-hand margin of (a). Patterns essentially identical with those illustrated were observed after similar analyses by t.l.c. of two other portions of the ganglioside fraction of each tissue and tumour indicated above.

Table 3. *Distribution of sialic acid in the principal gangliosides of lung and lung tumours and the total lipid-bound sialic acid contents of these tissues.*

The distribution of sialic acid in the principal ganglioside zones was determined by the method of MacMillan & Wherrett (1969). These results were summed to yield the total lipid-bound sialic acid content of the tissues, expressed as μg of sialic acid/mg of protein. The results are the means of duplicate analyses of each tumour; the lung data represent the means and standard errors of duplicate determinations on each of four different specimens. The variation between the results of the duplicate determinations was less than 10% for all of the samples analysed.

Tissue	G _{M3}	G _{M2} to G _{M1}	G _{D1a} to G _T	Total lipid-bound sialic acid
Lung	0.29 $\pm$ 0.03	0.11 $\pm$ 0.01	0.11 $\pm$ 0.02	0.50 $\pm$ 0.03
Squamous-cell tumour A	0.16	0.06	0.02	0.24
Squamous-cell tumour B	0.21	0.03	0.07	0.31
Squamous-cell tumour C	0.23	0.14	0.14	0.51
Squamous-cell tumour D	0.10	0.03	0.05	0.18
Adeno-squamous-cell carcinoma A	0.20	0.20	0.28	0.68
Adeno-squamous-cell carcinoma B	0.67	0.10	0.22	0.99
Adenocarcinoma	0.40	0.16	0.07	0.63
Large-cell carcinoma	0.35	0.12	0.22	0.69
Oat-cell carcinoma	0.37	0.10	0.32	0.79

amounts of *N*-acetylglucosamine; the corresponding gangliosides from normal lung did not contain this sugar. However, as all of these tumours may have been contaminated by polymorphonuclear leucocytes (see below), the possibility that the *N*-acetylglucosamine-containing species derived from these cells could not be discounted. In relation to this observation, analysis by t.l.c. of the gangliosides of the pneumonic lung showed that its ganglioside pattern was very similar to that of the control lung. However, analyses by g.l.c. of certain of the more complex gangliosides (corresponding in migration to G_{M1} and G_{D1a}) again indicated the presence of *N*-acetylglucosamine. Analyses of the gangliosides of two of

the preparations of polymorphonuclear leucocytes by t.l.c. revealed that only traces of gangliosides were detectable. The small amount of gangliosides obtainable from the preparations of polymorphonuclear leucocytes used thus did not permit direct examination of the question as to whether these cells possess certain *N*-acetylglucosamine-containing gangliosides.

Discussion

The above studies have provided new information on the neutral glycosphingolipids of both normal human lung tissue and of a number of different types

of human lung tumours. No previous studies of the glycolipids of normal human lung tissue appear to have been reported. The neutral glycosphingolipids present in pig lung have been analysed previously (Gallai-Hatchard & Gray, 1966); glucosyl-, lactosyl-, globotriaosyl- and globotetraosyl-ceramides were found to be the major components. Our studies also suggest that these four compounds are the principal neutral glycosphingolipids of human lung. All four of these neutral glycosphingolipids were detected in all of the lung tumours studied, in some cases in amounts corresponding approximately to those found in lung. The results of two previous investigations of the neutral glycosphingolipids of human carcinomata are of interest in relation to this finding. Firstly, Karlsson *et al.* (1974) performed a detailed analysis of the neutral glycosphingolipids of one renal carcinoma. In comparison with control human kidney, which contained mono-, di-, tri- and tetra-glycosyl-ceramides and sulphogalactosylceramide, the amounts of the three latter components were markedly reduced and the amount of lactosylceramide was markedly increased. One interpretation of these data is that the tumour tissue exhibited a partial block of the conversion of lactosylceramide to more complex glycosphingolipids. Secondly, Siddiqui *et al.* (1978) characterized the neutral glycosphingolipids of normal colonic mucosa and of colonic adenocarcinomata. The normal colonic mucosa contained a complex mixture of neutral glycosphingolipids, including glucosyl-, galactosyl-, lactosyl-, globotriaosyl-, globotetraosyl-, neolactotetraosyl- and sulphogalactosyl-ceramides. The tumours exhibited elevated amounts of lactosyl- and sulphogalactosyl-ceramides and a decreased amount of galactosylceramide. However, the other more complex neutral glycosphingolipids were present at the same concentrations in the carcinomata as in the normal mucosa, arguing against the possibility that the increase in lactosylceramide was simply due to a block in its conversion into more complex glycosphingolipids. The results of the present studies of the neutral glycosphingolipids of human lung tumours generally resemble those described by Siddiqui *et al.* (1978), in that no clear evidence of a block in the biosynthesis of the more complex neutral glycosphingolipids was detected as evaluated by their detection in appreciable amounts by both t.l.c. and g.l.c. Certain of the tumours did show elevated amounts of lactosylceramide (see below), but sulphogalactosylceramide was not a major component of either the control tissue or the tumours (as evaluated by comparison by t.l.c. with an authentic standard of this compound).

The finding that some of the tumours analysed in this study contained the putative lactotriaosylceramide species and also neolactotetraosylceramide was of interest, particularly as the former compound may be intimately related to the biosynthesis of

certain of the blood-group substances (Ando *et al.*, 1976). However, the observation that the other tumours did not exhibit these two glycosphingolipids raised the possibility that these compounds could have derived from non-tumour cells contaminating certain of the tumours. For reasons described above, the possibility that these compounds were derived from polymorphonuclear leucocytes was considered. Our studies confirmed the previous demonstration of neolactotetraosylceramide in polymorphonuclear leucocytes (Wherrett, 1973), revealed the presence in these cells of the putative lactotriaosylceramide and also showed that both of these glycosphingolipids were present in a specimen of lung containing a heavy infiltration of polymorphonuclear leucocytes. These findings, along with the overall variability of the occurrence of these lipids in the tumours analysed, lead us to propose that these particular neutral glycosphingolipids reflected the presence of contaminating polymorphonuclear leucocytes in the tumours that contained these compounds. Unfortunately, no independent biochemical marker (such as an enzyme activity present in polymorphonuclear leucocytes and not in lung tumour cells) was used in this study to assess the possible extent of contamination of the tumours by these cells, as the possible importance of such a parameter was not realized until after the tumours had been analysed. Thus we cannot exclude the possibility that certain of the tumour cells did actually contain these particular neutral glycosphingolipids. The use of immunological approaches, employing, for example, fluorescent-labelled antibodies to neolactotetraosylceramide and to the putative lactotriaosylceramide (cf. Marcus, 1976), might help to confirm or deny this possibility. Two further points concerning these particular neutral glycosphingolipids merit brief discussion. The identity of neolactotetraosylceramide was established in this study by the finding that the compound, when isolated from certain of the tumours, showed a reaction of complete identity with an authentic standard when tested against the specific antiserum used. However, the identity of the glucosamine-containing triglycosylceramide species has only been partially established, on the basis of chromatographic migrations compared with a standard of lactotriaosylceramide and on the basis of its carbohydrate composition. It is proposed that the compound is lactotriaosylceramide, rather than some other isomer, principally because the former compound is so directly related to neolactotetraosylceramide with respect to the latter's pathways of metabolism. The presence of the putative lactotriaosylceramide in polymorphonuclear leucocytes has not apparently been reported before. Lactotriaosylceramide has been previously detected in abnormal (Joseph & Gockerman, 1975) and normal (Ando *et al.*, 1976) human erythrocytes.

Another finding concerning the neutral-glycosphingolipid patterns of certain of the tumours lent some additional support to our hypothesis that contamination by polymorphonuclear leucocytes was contributing to their content of glycosphingolipids. This was the observation that all of the tumours exhibiting the putative lactotriaosylceramide and neolactotetraosylceramide also contained elevated amounts of lactosylceramide. Human polymorphonuclear leucocytes have previously been shown to contain appreciable amounts of this glycosphingolipid (Miras *et al.*, 1966). Our analyses confirmed this report and demonstrated that lung tissue contaminated by these cells exhibited relatively high amounts of this compound. It is pertinent to recall that one of the earliest abnormalities of glycosphingolipids reported in tumour cells was the presence of elevated amounts of lactosylceramide (reviewed by Rapport, 1969). In general, this finding has not been a consistent one in cultured transformed cells (Hakomori, 1975a). However, in both of the studies previously cited of the neutral glycosphingolipids of human carcinomata (Karlsson *et al.*, 1974; Siddiqui *et al.*, 1978), elevated amounts of this particular glycosphingolipid were reported. The present results suggest that one explanation for the detection of elevated amounts of lactosylceramide in tumours growing *in vivo* may be contamination by polymorphonuclear leucocytes. It is unlikely that this is the only cause of this phenomenon. Nevertheless, on first principles, it seems reasonable to assume that if no biosynthetic block exists in the conversion of lactosylceramide (as monitored by the criterion previously mentioned), there would be no apparent reason for an accumulation of lactosylceramide, unless the galactosyl hydrolase involved in its catabolism (not measured here) was deficient. What is evident is that some of the lung tumours did not show elevated amounts of this glycolipid, so that this alteration is certainly not a consistent finding in all human tumours growing *in vivo*.

The presence of a relatively complex profile of gangliosides in normal human lung tissue has not been reported previously. In their study of the glycolipids of pig lung, Gallai-Hatchard & Gray (1966) described the presence of one simple ganglioside, presumably G_{M3}. The ganglioside profiles of the tumours studied here were also complex, although certain of the tumours did show decreases in the more complex gangliosides observed in lung. It is unlikely that contamination by polymorphonuclear leucocytes contributed significantly to the ganglioside profiles of certain of the tumours, as analyses of pneumonic lung revealed that the ganglioside profile of this tissue was very similar to that of normal lung. However, even if contamination by these cells did contribute to the ganglioside profiles of certain of the tumours, it should be noted that the four tumours that contained

little or none of the neutral glycosphingolipids thought to be contributed by polymorphonuclear leucocytes also exhibited complex patterns of gangliosides. The finding that lung tumours exhibit relatively complex ganglioside profiles is similar to observations made on Morris hepatomas grown *in vivo* (Siddiqui & Hakomori, 1970; Cheema *et al.*, 1970). The ganglioside profiles of human gastric (Keränen *et al.*, 1976) and colonic (Siddiqui *et al.*, 1978) adenocarcinomata have been studied previously; in both of these reports, little change was also noted in the ganglioside profiles of the tumours as compared with the control tissues.

Despite the fact that the tumours analysed by us did not exhibit notable simplifications of the profiles of the two classes of glycosphingolipids analysed, it is not justifiable from the observations made here to conclude that alterations of glycosphingolipids could not be of significance in explaining some of the abnormal biological properties displayed by lung tumour cells. Consideration of the following points will clarify this statement. (1) The suitability of the use of whole lung tissue as the control tissue can be questioned, as most lung tumours are believed to originate from bronchial epithelial cells (Millard, 1977). We have not found it possible to obtain sufficient bronchial mucosa to use as the control tissue. However, the general similarities of the patterns of neutral glycosphingolipids and gangliosides of the lung and lung tumours encourage us to believe that the use of the former tissue may not have been inappropriate. Nevertheless, the possibility cannot be overlooked that bronchial epithelium may contain more complex neutral glycosphingolipids and gangliosides than were detected in the lung tissue analysed, and that these components were missing from the tumours. (2) No analyses of fucose-containing or other complex glycosphingolipids directly related to the blood-group substances were performed here. Siddiqui *et al.* (1978) reported a marked decrease or total absence of glycolipid-associated blood-group activity in all of the colonic carcinomata studied by them. In addition, two other complex glycolipids, possible precursors of the blood-group substances, were detected in the tumours but not in normal colonic mucosa. Abnormalities of other similar glycosphingolipids related to the blood-group substances had previously been reported in other tumours (reviewed by Hakomori, 1975b). (3) Momoi *et al.* (1976) have shown that a large number of minor gangliosides can be resolved from certain tissues, by using appropriate techniques. It is possible that such components have been overlooked here. Moreover, no structural analyses were performed by us on the complex gangliosides present in lung and the tumours; it is possible that such analyses might have revealed significant structural differences between lung and tumour gangliosides. (4) In

addition, no studies were performed here on the distribution of the constituent glycosphingolipids in the plasma-membrane fractions of the tumour cells, by techniques such as labelling with galactose oxidase; use of this approach has shown that some transformed cells may show alterations in the distribution of certain glycosphingolipids in the plasma membrane as compared with normal cells (Gahmberg & Hakomori, 1975).

It does appear justifiable, however, to conclude that, regardless of their precise extent, the alterations in glycosphingolipid profile that are shown by human lung and other tumours are less marked than those detected in many cultured transformed cells (Hakomori, 1975a). With respect to the design of possible experiments to test whether alterations of glycosphingolipid profiles are really of significance in contributing to the abnormal biological behaviour of human tumour cells growing *in vivo*, the evidence to date would suggest that particular attention should be focused on the possible involvement of the more complex members of this group: the higher gangliosides and the blood-group-associated compounds. It has not been established whether the more marked alterations in glycosphingolipid composition exhibited by cultured transformants reflect in part the relatively abnormal conditions of tissue culture. Clonal variation has been shown to be an important consideration in assessing the possible significance of alterations of glycosphingolipid profile in cultured cells (Sakiyama *et al.*, 1972; Yogeewaran *et al.*, 1972). It is also relevant to note that viral transformants appear to show more prominent alterations of glycosphingolipid profile than do chemically or 'spontaneously' transformed cells (Hakomori, 1975a). Whether this situation has any relationship to the observations that human tumour cells growing *in vivo* appear to show at most rather limited changes of glycosphingolipid pattern can at present only be the subject of speculation.

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