PRELIMINARY EVIDENCE FOR NOVEL FUCOSE-CONTAINING LIPIDS IN NORMAL AND MURINE SARCOMA VIRUS-TRANSFORMED RAT CELLS

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Summary — Two fucose-containing lipids with long chain base as their apolar moiety are described. These compounds were shown to possess a free amino group and a net positive charge by derivatization with specific amino reagents and by cation exchange column chromatography. The presence of long chain base was demonstrated by periodate-NaB[^{3}H]$_4$ treatment to yield long chain alcohol and by [^{3}H]-dansylation followed by hydrolysis to yield dansyl-long chain base.

The incorporation of isotopically-labeled fucose into four chromatographically distinct, lipid extractable components in a variety of cultured cells (1) has been demonstrated in this laboratory. Incorporation of [^{3}H] or [^{14}C]-fucose into the least chromatographically mobile, presumably most complex fucolipid, FL IV, is markedly decreased in oncornavirus-transformed cell lines and in cultured human tumor cells (2-4). Often the decreased incorporation is accompanied by a build-up in a chromatographically more mobile fucolipid, FL III. In this communication evidence will be presented to indicate that FL III and FL IV are novel, fucose-containing sphingolipids.

MATERIALS AND METHODS

Cell growth and harvesting. The cells employed were NRK and MSV-NRK (2). Cell cultures were periodically examined for mycoplasma. Cells were grown in Eagle's medium plus 10% fetal calf serum (v/v) and supplemented with either [^{3}H]-6-fucose (5 μCi/ml; 13.1 Ci/mmole; New England Nuclear Corp.) or [^{14}C]-fucose (0.5 μCi/ml; 54 mCi/mmole; New England Nuclear Corp.). Cells were harvested and fucolipids extracted as previously described (1).

Purification of FL III and FL IV. The total lipid extract was taken to dryness under N_2, resuspended in $CHCl_3$-CH_3OH (2:1, v/v) and partitioned accord-

Abbreviations used are: FL, fucolipid; MSV, murine sarcoma virus; NRK, newborn rat kidney cells; MSV-NRK, NRK cells transformed by and producing murine sarcoma-murine leukemia virus complex; TNBSA, trinitrobenzene sulfonic acid; dansyl, 1-dimethyl-naphthalene-5-sulfonyl-chloride.

ing to the method of Folch et al. (5). FL III and FL IV were quantitatively recovered in the aqueous phase. This phase was dried, resuspended in $CHCl_3$-CH_3OH-pyridine-H_2O (40:56:12:2, by vol.) and chromatographed in solvent I [2-propanol-NH_4OH-H_2O (7:2:1, by vol.)] followed by chromatography in solvent II [$CHCl_3$-CH_3OH-H_2O (60:35:8, by vol.)] on silica gel plates (Q5, Quantum Industries). The fucolipids were located by autoradiography if [^{14}C]-labeled or by use of a radiochromatogram scanner if [^{3}H]-labeled and were eluted with solvent I. Fucolipid preparations examined for the presence of long chain base were further purified. The samples were dried, resuspended in H_2O, and applied to cation exchange columns of Bio-Rad AG 50, H^+ form. To avoid breakdown, chromatography on AG 50 was performed rapidly and the eluate maintained at ice bath temperature. The fucolipids were eluted from the AG 50 columns with 0.5 N NH_4OH, concentrated by rotary evaporation, and re-chromatographed in 2 dimensions: 1st dimension, isobutyric acid-NH_4OH-H_2O (59:4:39, by vol.); 2nd dimension, solvent II. Several areas lacking fucose radioactivity were scraped from the plate and designated as blanks.

Amino group derivatization. N-acetylation was carried out by the method of Marcus et al. (6). Derivatization using dansyl-chloride was carried out by overnight incubation, at 22°C, of 22 mM dansyl-chloride or [^{3}H]-dansyl-chloride (18.1 Ci/mmole; 1.5 mg/ml; New England Nuclear) in acetone with an equal volume of the purified FL III or FL IV in 0.1 M $NaHCO_3$. Dansylated psychosine, sphingosine, dihydrosphingosine, glucosamine, galactosamine and valine were prepared in an analogous manner. Dansyl FL III was isolated by chromatography on Q5 plates in solvent II. Further purification was accomplished by chromatography in $CHCl_3$-CH_3OH-H_2O (40:10:1, by vol.), followed by chromatography in $CHCl_3$-CH_3OH-H_2O (40:20:1.2, by vol.). Derivatization with TNBSA was accomplished by the method of Gordesky and Marinetti (7). The above procedures did not result in alterations in the chromatographic mobility of authentic lactosylceramide or galactosylceramide.

Periodate-[^{3}H]-borohydride treatment. Purified FL III was subjected to periodate oxidation followed by [^{3}H]-borohydride reduction by the method of Carter and Hirschberg as modified by Smith and Lester (8). The radioactivity incorporated into long chain alcohols was proportional to the initial concentration of standard long chain bases. Authentic sphingosine, dihydrosphingosine, phytosphingosine, lactosylceramide, galactosylceramide and sphingomyelin were processed in an analogous manner to serve as controls.

Acid hydrolysis. To verify that the radioactivity incorporated into the fucolipids was fucose, intact FL III and FL IV were subjected to mild acid hydrolysis followed by chromatographic identification of the hydrolysis product (1). Hydrolysis of [^{3}H]-dansyl-[^{14}C]-FL III was carried out in 1.0 N methanolic HCl at 80°C for 30 min. The hydrolysate was adjusted to a $CHCl_3$-CH_3OH ratio of 2:1, by vol., and 0.1 volume of H_2O was added. The upper aqueous phase was removed and the lower organic phase, i.e., dansyl-long chain base fraction, was washed with CH_3OH-H_2O (1:1, by vol.) to remove residual acid.

Chemicals. Psychosine was from Serdary Research Laboratories, Inc. (Canada). All other lipids were from Applied Science Laboratories. Non-radioactive dansyl-chloride was purchased from Pierce Co. TNBSA was from Sigma; non-radioactively labeled $NaBH_4$ was from Fisher Chemicals; and [^{3}H]-$NaBH_4$ (10.5 Ci/mmole, vol.) was from Amersham/Searle.

RESULTS

A number of fucolipids have been characterized (9-13). These compounds vary in the length of the carbohydrate chain but all presumably contain ceramide as the lipid moiety. Initial characterization of FL III and FL IV indicated similar general features with previously described fucolipids (9-13), i.e., fucose-containing, mild-alkali stable, H_2O soluble, and do not bind to DEAE-cellulose. However, as shown in Table 1, FL III and FL IV have quite dissimilar chromatographic properties when compared with authentic blood group fucolipids. The low chromatographic mobility of FL III and FL IV in a neutral pH solvent and the increased mobility in a basic pH solvent suggested the possibility that these compounds possess a net positive charge at neutral pH. To examine this possibility FL III was chromatographed on AG 50 cation-exchange columns and was observed to bind to the resin (Table 2). A similar elution pattern was obtained with FL IV.

To examine whether or not the positive charge was due to a free amino

TABLE 1. Comparison of the chromatographic mobility of blood group determinant fucolipids A^a and A^b with [^{14}C]-labeled fucolipids III and IV

Fucolipid	Rf value	
	Solvent I (2-propanol-NH_4OH-H_2O, 7:2:1, by vol.)	Solvent II ($CHCl_3$-CH_3OH-H_2O, 60:35:8, by vol.)
FL A^a *	0.17	0.54
FL A^b *	0.11	0.26
FL III **	0.42	0.11
FL IV **	0.31	0.06

Fucolipids A^a and A^b were the generous gift of Dr. S-I. Hakomori, Univ. of Washington, Seattle. * Position located by orcinol spray. ** Position located by autoradiography of [^{14}C]-fucose labeled components.

group, FL III and FL IV were reacted with amino derivatizing reagents, i.e., acetic anhydride, dansyl-chloride and TNBSA. Table 3 indicates that the chromatographic properties of FL III and FL IV are altered following the various N-amino-derivatization procedures. In addition, N-acetylated FL III and FL IV were not retained on AG 50. These results are consistent with the interpreta-

TABLE 2. Chromatography of FL III on AG 50 (H^+) column

	Counts/min	% recovered
Original sample	93,100	100
H_2O eluate 1 *	3,300 **	3.5
H_2O eluate 2	100 **	0.1
H_2O eluate 3	0	0
0.5 N NH_4OH eluate 1	81,650	87.7
0.5 N NH_4OH eluate 2	0	0

* Each H_2O eluate was 5.0 ml; each 0.5 N NH_4CH eluate was 10.0 ml. ** This radioactivity had the chromatographic properties of fucose and probably indicates a partial breakdown. See Methods section for experimental procedure.

TABLE 3. Chromatographic mobility of FL III and FL IV following N-acetylation, dansylation and derivatization with TNBSA

	Rf values					
	− N-acetylation	+ N-acetylation	− Dansyl	+ Dansyl	− TNBSA	+ TNBSA
FL III	0.11	0.28	0.11	0.61 (0.72)*	0.11	0.55 (0.66)
FL IV	0.06	0.28	0.06	0.50 (0.55)	0.06	0.50 (0.66)

Samples were derivatized as described in the Methods section and were chromatographed on thin-layer plates in solvent II. Samples indicated as −reagent were incubated under identical conditions except for the absence of the derivatizing reagent.
* A second minor peak was observed at the Rf values given in parentheses.

tion that FL III and FL IV contain an unsubstituted amino group.

Galactosyl-sphingosine (psychosine), a glycolipid lacking an amide-linked fatty acid, has been isolated from human tissues (14). If the fucolipids under study contain as the lipid moiety a long chain base with a free amino group, similar to psychosine, then periodate oxidation followed by [^{3}H]-borohydride reduction should yield [^{3}H]-fatty alcohol. Because of the very small amount of fucolipid per cell, relatively large amounts of cells were needed for examination of the lipid moiety. Hence, these studies were carried out using FL III which was obtained from MSV-NRK cells. Following direct periodate-[^{3}H]-$NaBH_4$ treatment two chromatographically distinct compounds with approximately equal amounts of [^{3}H]-radioactivity were observed in the long chain alcohol fraction (Table 4). The number of pmoles of long chain alcohol approximated the number

TABLE 4. Chromatographic mobility of long chain alcohols obtained from periodate oxidation-[^{3}H]-borohydride reduction of FL III

Component	Rf values			
	Solvent 1	Solvent 2	Solvent 3	Solvent 4
Sphingosine product*	0.64	0.27	0.21	0.43
Dihydrosphingosine product	0.79	0.39	0.26	0.46
Phytosphingosine product	0.79	0.39	0.26	0.46
Hexadecanol	0.79	0.39	0.26	0.46
Tetradecanol	0.79	0.39	0.26	0.46
Glycosyl-sphingosine (psychosine) product*	0.64	0.27	0.21	0.43
Fucolipid product A	0.64	0.27	0.21	0.43
Fucolipid product B	0.79	0.39	0.26	0.46

Solvent systems: 1, hexane-diethyl ether-acetic acid (30:70:1, by vol.); 2, hexane-diethyl ether-acetic acid (70:30:1, by vol.); 3, benzene-chloroform-acetic acid (90:10:1, by vol.); and 4, petroleum ether-diethyl ether-acetic acid (85:15:2, by vol.). * An additional minor component (~5%) which had an Rf value similar to hexadecanol was observed. For periodate treatment and borohydride reduction, see Methods section.

of pmoles of starting FL III. This estimation was based on the amount of radioactivity incorporated into FL III and into long chain alcohol. FL product A is apparently an unsaturated long chain alcohol, leading to the tentative conclusion that the parent lipid contains a sphingosine-like base. Because the chromatographic systems employed did not resolve saturated long chain alcohols of varying chain lengths, we could not establish whether the other long chain alcohol, i.e., FL product B, was derived from a phytosphingosine-like or a dihydrosphingosine-like long chain base. To more directly examine the location of the amino group, [^{14}C]-FL III was completely derivatized with [^{3}H]-dansyl, purified, acid hydrolyzed and the dansylated hydrolysis product identified. Table 5 indicates that the hydrolysis product has comparable chromatographic properties to dansyl-long chain base. The possibility of other dansylated hydrolysis products, i.e., amino acid or aminosugar derivatives, in the hydrolysate was examined and none were observed.

TABLE 5. Rf values of [^{3}H]-dansyl long chain base fraction obtained following acid hydrolysis of dansylated FL III

Parent compound	Rf values		
	Solvent A	Solvent B	Solvent C
[^{3}H]-dansyl-[^{14}C]-FL III *	0.51	0.76	0.23
Dansyl-psychosine **	0.51	0.76	0.23
Dansyl-sphingosine **	0.51	0.76	0.25
Dansyl-dihydrosphingosine **	0.49	0.76	0.20

For conditions of dansylation, purification and hydrolysis, see Methods section. Solvent systems: A, ethyl acetate-cyclohexane (3:2, by vol.); B, $CHCl_3$-CH_3OH-H_2O (90:10:1, by vol.); and C, hexane-diethyl ether-H_2O (30:70:1, by vol.). * Dansyl-psychosine was added as carrier in the [^{3}H]-dansyl-[^{14}C]-FL III hydrolysis mixture and the radioactive and fluorescent hydrolysis products comigrated in all 3 solvents. ** Identification of the standard dansylated long chain bases as well as the carrier dansylated psychosine was accomplished by ultraviolet fluorescence.

DISCUSSION

In this study evidence has been presented that FL III and FL IV have a free amino group. In addition, the amino group of FL III was shown to be located on a long chain base. Since FL III appears to be the metabolic precursor of FL IV (3), it is reasonable to suggest that the apolar moiety of FL IV is also a long chain base. The possibility that FL III and FL IV are produced during the extraction procedure cannot be completely ruled out. However, we doubt this since the extraction procedure is relatively gentle, similar components are consistently observed in a wide variety of cell lines, and we have been unable to detect other psychosine-like glycolipids in MSV-NRK or NRK cells. Studies employing larger quantities of FL III and FL IV for use with gas liquid chromatography and mass spectroscopy have been initiated to definitively establish the lipid and polar portion of these compounds. Sphingolipids, without fatty acid, are uncommon and although galactosylsphingosine and glucosylsphingosine have been reported, it is not entirely clear whether they are intermediates in the metabolism of glycosylceramide. In contrast, FL IV is apparently a relatively stable end-product of fucolipid metabolism in normal cells (3). Because of the unusual properties of these compounds, their wide distribution in cultured mammalian cells and their altered synthesis following transformation, it is possible that they play a unique role in membrane events associated with transformation.

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