NOVEL FUCOSE-CONTAINING COMPONENTS FROM RAT TISSUES

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Summary—— Four low-molecular-weight fucosylated compounds have been isolated from rat tissues. Three of these compounds have not been described previously and are composed of: serine and fucose; serine, fucose and glucose; and threonine and fucose. The fourth compound has the same amino acid-carbohydrate composition, i.e., threonine, fucose and glucose, as a component isolated from normal human urine (9).

Recent studies in this laboratory have focused on the differences in incorporation of [^{3}H]- or [^{14}C]-fucose into two organic-extractable, fucose-labeled components, i.e., FL3 and FL4, in normal versus oncogenically transformed cells (1-4). Initial characterization indicated that, in addition to being organic-extractable, these components were pronase-resistant, mild alkali stable, associated with the particulate fraction, resistant to phosphomonoesterase and phosphodiesterase, aqueous soluble, and contained a free amino group that was thought to be associated with a sphingosine-like base (5). In the current study, sufficient quantities of FL3 and FL4 were obtained from rat tissues for direct chemical analyses, and the results indicate that the compounds are unusual aminoacyl fucosides rather than sphingosyl fucosides and that FL3 contains threonine and fucose while FL4 contains threonine, fucose and glucose. Furthermore, two additional novel fucose-containing compounds, i.e., one containing serine and fucose and the other serine, fucose and glucose, have been isolated.

MATERIALS AND METHODS

Cell growth and harvesting. The cultured cells employed were MSV-NRK, i.e., murine sarcoma virus-transformed rat cells (2). Cells were grown in Eagle's medium plus 10% fetal calf serum (v/v) and supplemented with 5 μCi/

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ml [^{3}H]-6-fucose (25 Ci/mmole; Amersham Searle Corp.). Cells were harvested and extracted as previously described (4).

Purification of fucose-labeled components from rat tissue. Eighty Sprague-Dawley rats (4-6 weeks old), five of which were injected intraperitoneally with 50 μCi (in 1.0 ml sterile saline) of [^{14}C]-fucose (60 mCi/mmole) 12 hr prior to sacrifice, were killed and the intestines, kidneys, livers and brains were removed, rinsed in ice-cold H_2O, minced and extracted essentially by the method of Folch et al. (6). [^{3}H]-FL3 and [^{3}H]-FL4 extracted from MSV-NRK cells was added to the tissue extract and the combined sample was dried, resuspended in $CHCl_3$-CH_3OH (2:1, v/v) and partitioned according to the method of Folch et al. The aqueous phase was chromatographed on AG50 H^+ and the compounds of interest were eluted with NH_4OH as previously described (5). The material was purified by thin-layer chromatography (TLC) (Q-5 plates, Quantum Industries, Fairfield, N.J.) sequentially in the following solvents: (A) $CHCl_3$-CH_3OH-H_2O (60:35:8, by vol); (B) 2-propanol-NH_4OH-H_2O (7:2:1, by vol); (C) $CHCl_3$-CH_3OH-NH_4OH (40:80:25, by vol); (D) 1-butanol-pyridine-H_2O (6:4:3, by vol); (E) $CHCl_3$-CH_3OH-acetone-acetic acid-H_2O (140:35:25:80:9, by vol). Solvent B was used to elute the material from the TLC plate prior to chromatography in each subsequent solvent. The final step was silicic acid column chromatography using batchwise elution with $CHCl_3$, acetone and CH_3OH. All the FL components eluted with CH_3OH.

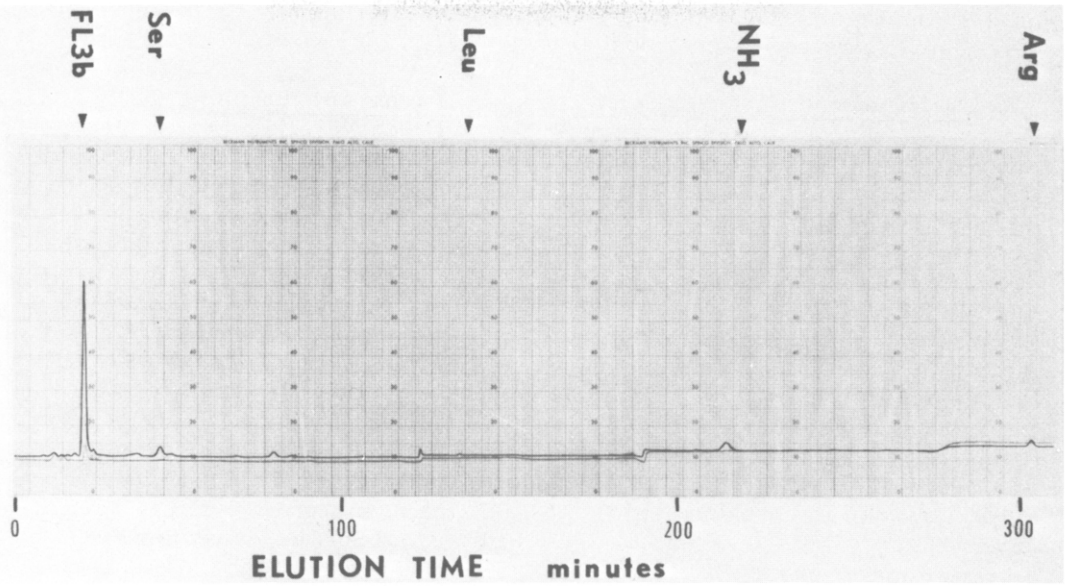
The position of the compounds of interest and of other material was monitored by combined use of: radioactivity, orcinol reagent, ninhydrin reagent, I_2-vapor, and UV absorption. As judged by these criteria, solvent systems A through E were all necessary to separate the compounds of interest from other components.

Gas-liquid chromatography (GLC) analysis. Samples were prepared for GLC essentially by the method described by Laine et al. (7). The dried samples were methanolyzed in anhydrous 1.0 N methanolic HCl at 80°C for 20 hr, then 0.1 μmole of mannitol was added, the sample extracted three times with n-hexane, neutralized with Ag_2CO_3, and re-N-acylated with acetic anhydride. After pelleting and washing thrice with CH_3OH, the combined supernatants were dried in vacuo over KOH for 12-16 hr and silylated, at ambient temperature for 30 min, in hexamethyldisilazane-trimethylchlorosilane-pyridine (1.3:0.8:1.0, by vol) (8).

Gel filtration. Samples were analyzed on Sephadex G-10 columns (0.8 x 70 cm) eluted with 0.2 M NH_4-acetate (pH 7.2) with a flow rate of ~0.13 ml/min. Standard curves were constructed with amino acids (leucine, serine, threonine, lysine), peptides (leucyl-valine, leucyl-leucine) and saccharides (fucose, lactose, stachyose, raffinose).

RESULTS

The purification of [^{14}C]-fucose-labeled material from rat tissue is described in the Methods section. As previously shown with material from cultured cells (5), chromatography in solvent B separated FL3 from FL4. How-



ELUTION TIME minutes

FIG. 1. Chromatography of purified FL3b. After purification, FL3b was resuspended in 0.11 N Na-citrate, pH 3.2, and chromatographed on a Beckman 121 amino acid analyzer using a single column, physiological fluid program. The arrows indicate the position of authentic standards.

ever, when the components from rat tissue were chromatographed in solvent C, FL3 and FL4 each separated into two compounds, i.e., FL3a and FL3b and FL4a and FL4b. Contrariwise, the exogenously added material from cultured cells, i.e., [^{3}H]-FL3 and [^{3}H]-FL4, quantitatively co-migrated with [^{14}C]-labeled FL3a and FL4a, respectively. Moreover, comparable results were obtained when [^{3}H]-fucose was used for in vivo labeling.

When the purified compounds were chromatographed in solvent F, i.e., butanol-propionic acid-H_2O (6:3:4, by vol), they yielded a single radiolabeled, ninhydrin-positive, orcinol-positive, I_2-vapor-negative, non-UV-absorbing compound. The purity of intact FL3a, FL3b and FL4a was further assessed by chromatography on a Beckman 121 amino acid analyzer, and each yielded a single major ninhydrin-positive component as typified by FL3b (fig. 1). A portion of each of the highly purified compounds was analyzed by GLC after

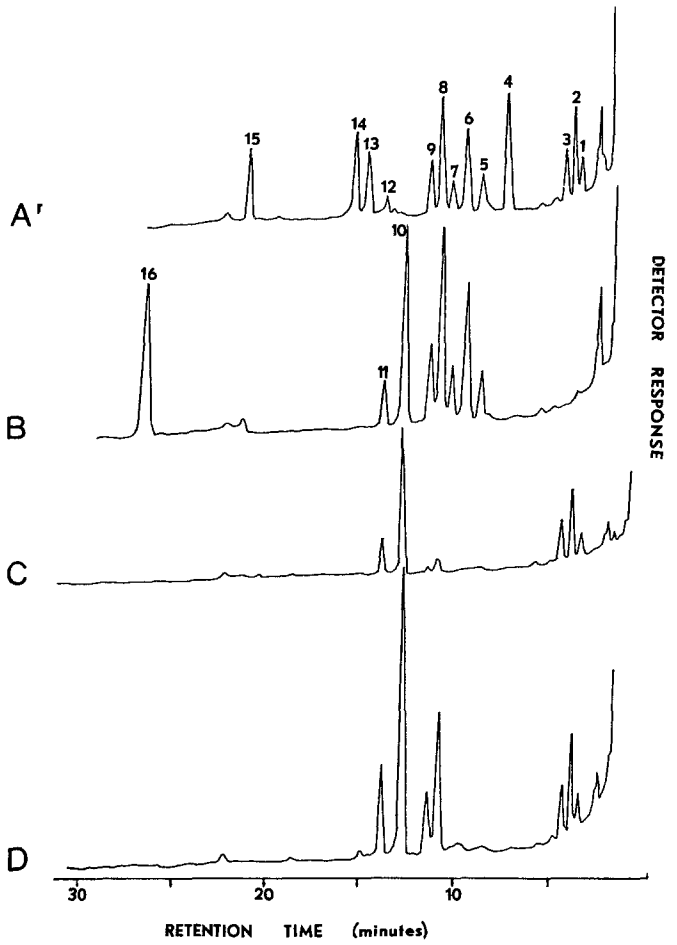


FIG. 2. Gas-liquid chromatography of FL3b and FL4a. For details of acid methanolysis and derivatization of samples, see Methods section. (A) Top profile: Equimolar standard mixture of monosaccharides. (B) Second profile from top: Lactosylceramide. (C) Third profile from top: FL3b. (D) Bottom profile: FL4a. Peaks correspond to the following compounds: 1,2,3, fucose; 4, arabinitol; 5,6,7, galactose; 8,9, glucose; 10,11, mannitol; 12, 13, N-acetylgalactosamine; 14, N-acetylglucosamine; 15, N-acetylneuraminic acid; and 16, dihydrosphingosine. 1-2 μl of each sample was injected onto 6-foot columns packed with 3% CV-1 on 80/100 Supelcoport in a Packard Becker model 419 dual column gas chromatograph with flame ionization detector. N_2 flow rate (effluent), 30 ml/min. Injector and detector temperatures, 270°C. Temperature program: Temperature maintained at 160°C for 5 min, then raised 5°/min to 250°C, and maintained at 250°C for 15 min. Peak areas were integrated using a Spectra-Physics Autolab System I computing integrator.

acid methanolysis and appropriate derivatization. Both FL3a and FL3b (fig. 2C) yielded peaks with retention times comparable to fucose. FL4a (fig.

TABLE 1. Amino acid analysis of aminoacyl fucosides

Compound	Retention time (minutes)	
	Before hydrolysis	After hydrolysis
FL3a	21.9	43.0
FL3b	23.6	46.2
FL4a	16.0	42.9
FL4b	ND	46.3
Serine*	—	46.8
Threonine*	—	43.3

Samples were hydrolyzed in 6.0 N HCl at 110°C for 24 hr, concentrated by flash evaporation, resuspended in a small volume (0.05-0.1 ml) of buffer (see fig. 1 for details). * The amino acids with closest retention times to serine and threonine are aspartic acid (40.6) and proline (65.9). ND = not done.

2D) and FL4b yielded peaks with retention times comparable to fucose and glucose. Neither a lipid moiety, i.e., sphingosine-type base, nor fatty acid, nor an amino sugar moiety, i.e., N-acetyl glucosamine, N-acetyl galactosamine or N-acetyl neuraminic acid, was detected in any of the samples.

A second aliquot was subjected to strong acid hydrolysis and chromatographed on a Beckman 121 automated amino acid analyzer. After hydrolysis the ninhydrin-positive peak that was observed with intact FL3a, FL3b and FL4a was no longer detectable and a ninhydrin-positive peak with a comparable retention time to threonine for FL3a and FL4a and to serine for FL3b was observed (table 1). Because of the small amount of FL4b available, i.e., ~10 nmoles total, this compound was not examined on the amino acid analyzer prior to hydrolysis. Following hydrolysis, FL4b yielded a compound with a comparable retention time to serine. A summary of the molar ratios of carbohydrate and amino acid based on the combined data from GLC and amino acid analyzer is summarized in table 2.

Gel filtration on Sephadex G-10 was utilized to estimate the molecular

TABLE 2. Molar ratios of carbohydrate and amino acids of FL3a, FL3b, FL4a, and FL4b

Compound	nmoles *				Fuc / Glc / Thr / Ser			
	Fucose	Glucose	Threonine**	Serine**				
FL3a	91	0	84	0	1.1	0	1.0	0
FL3b	137	0	0	120	1.1	0	0	1.0
FL4a	53	56	46	0	1.2	1.2	1.0	0
FL4b	9	11	0	8	1.1	1.4	0	1.0

For details of analysis of carbohydrate and amino acids, see fig. 2 and table 1, respectively. * The nmoles of amino acid and carbohydrate represent the total purified material recovered from the rat tissues. As judged by exogeneously added FL3a and FL4a from cultured cells, recovery of FL3a from rat tissue was approximately 16.0% and recovery of FL4a was approximately 10.0%. **The amount of threonine and serine is not corrected for breakdown (~10%) due to strong acid hydrolysis.

size of FL3a and FL4a. Because appropriate standards, i.e., aminoacyl glycosides, were not available, it was only possible to estimate a molecular weight range. However, based on the maximum estimated molecular weight, i.e., 440 for FL3a and 560 for FL4a, it does not appear as though these compounds contain two residues of amino acid per molecule.

DISCUSSION

The isolation of several novel aminoacyl fucosides has been described in this study. Sufficient material was available to monitor the purification by chemical means, and it was observed that additional thin-layer chromatographic solvents, i.e., C, D and E, beyond those utilized in the isolation of isotopic levels of FL3a from cultured cells were needed to obtain the compound free from extraneous ninhydrin-, orcinol- and I_2-vapor-positive material. Thus, it seems reasonable to speculate that the sphingosine-containing compound isolated in the previous study (5) was co-purified with isotopically labeled FL3a.

The results of the present study indicate the presence of at least three novel low-molecular-weight, fucosylated components which contain: threonine and fucose (FL3a), serine and fucose (FL3b) and serine, fucose and glucose (FL4b). A fourth component, i.e., FL4a, has an amino acid and carbohydrate composition comparable to a compound isolated by Hallgren et al. (9) from normal human urine, i.e., threonine, fucose and glucose. The urinary component is unusual in that not only is the fucose residue linked to threonine, but it is also internal, i.e., 3-O-β-D-glucopyranosyl-α-L-fucopyranosyl-L-threonine. The compounds described in this paper may be structurally related to the urinary aminoacyl fucoside.

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