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EFFECTS OF A MEMBRANE SUGAR ANALOGUE, 6-DEOXY-6-FLUORO-D-GALACTOSE, ON THE L1210 LEUKEMIC CELL ECTOSIALYLTRANSFERASE SYSTEM

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Abstract—In L1210 leukemia cells, 6-deoxy-6-fluoro-D-galactose specifically inhibited the incorporation of [^{3}H]-D-galactose, while that of other precursors of glycoconjugate biosynthesis, including mannose and glucosamine, was unaffected. The activation of [6-^{3}H]-6-deoxy-6-fluoro-D-galactose to a nucleotide sugar was similar to that found for [^{3}H]-D-galactose. The incorporation of either sugar after 1 hr was visualized by electron microscopic autoradiography to be in the Golgi region. Treatment of L1210 cells with 6-deoxy-6-fluoro-D-galactose *in vitro* or *in vivo* resulted in a specific, dose- and time-dependent decrease in the activity of cell surface sialyltransferase (ectosialyltransferase) but not of 5′-nucleotidase, a plasma membrane marker enzyme. The decrease in ectosialyltransferase activity appeared to be selective and is suggested to be due to structural modification of the cell surface galactoprotein acceptors for this enzyme. The data indicate that 6-deoxy-6-fluoro-D-galactose is an effective modifier of cellular glycoconjugate in that its incorporation into certain cell surface components results in a modification of plasma membrane structure and function.

Transformed cells can be distinguished from non-transformed cells on the basis of altered characteristics in cell surface glycoconjugates [1]. Agents which modify the synthesis of these membrane components may have potential as cancer chemotherapeutic agents provided that these modifications translate into reductions in tumor cell growth, invasiveness, metastasis and increased antigenicity [2, 3]. Analogues of membrane sugars may act in this manner, provided that they are functional analogues, capable of being incorporated into glycoconjugates destined for the cell surface.

The sugar analogue 2-deoxy-D-glucose has been shown to modify the tumor cell surface [4, 5], but it appears to act by blocking an early step in the processing of nascent oligosaccharide chains [6] which results in widespread, if not lethal, perturbations of cellular metabolism. By contrast, the sugar analogue 6-deoxy-6-fluoro-D-galactose (6-Fgal)† should, by virtue of the halogen at C-6 position [7], block the formation of $\alpha(2 \rightarrow 6)$ glycosidic linkage between sialic acid and D-galactose (gal). This particular effect could alter the attachment of sialic acid which appears to be important in the expression of the tumor cell phenotype. For example, the capacity of a certain mouse mammary tumor to synthesize cell surface sialoglycoproteins, such as epiglycanin [8], or of a rat mammary ascites tumor to synthesize membrane glycoprotein ASGP-1 [9], is associated with a loss of susceptibility of these tumors to allogeneic growth restraints *in vivo*. In rodents, a correlation also exists between the degree of cell surface sialylation and the capacity of a variety of tumors to metastasize [10]. Similarly, sublines of the murine B16 melanoma, selected for increased capacity for lung metastasis, also exhibited an increase in cell surface sialylation over that of the parent B16 line [11–13]. Therefore, agents which modify tumor cell surface glycoconjugates may have some potential as chemo- or immunotherapeutic agents.

While subtle modifications of cell surface components may ultimately manifest themselves as alterations in growth or metastasic behavior of tumors, the studies reported herein concern effects of 6-Fgal in L1210 leukemia cells. These cells have an unusually active sialyltransferase system (EC 2.4.99.1) at their cell surface and, therefore, offer an excellent model for sialic acid attachment *in situ* [14, 15] in that analogue-induced alterations in the enzyme or its acceptor molecule would affect overall activity of the ectosialyltransferase system and provide strong evidence for a specific alteration in cell surface function. A preliminary report of these studies has been published [16].

MATERIALS AND METHODS

6-Deoxy-6-fluoro-D-galactose and [6-^{3}H]-6-deoxy-6-fluoro-D-galactose. The sugar analogue 6-deoxy-

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† Abbreviations: 6-Fgal, 6-deoxy-6-fluoro-D-galactose; gal, D-galactose; man, D-mannose; gln, D-glucosamine; leu, L-leucine; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; TBA, thiobarbituric acid; PCA, perchloric acid; NANA, *N*-acetylneuraminic acid; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; MOPS, 3-(*N*-morpholino) propane sulfonic acid; and PTA, phosphotungstic acid.

6-fluoro-D-galactose was synthesized as described previously [17]. [6-^{3}H]-6-Deoxy-6-fluoro-D-galactose([^{3}H]-6-Fgal) was obtained by the tritiated borohydride reduction of a synthetic intermediate of 6-Fgal. 1,2:3,4-Di-*O*-isopropylidine-D-galactose was oxidized to the corresponding aldehyde, which was reduced with 25 mCi [^{3}H]$NaBH_4$ (sp. act. 222 Ci/mmole, New England Nuclear Corp., Boston, MA), followed by treatment with diethylaminosulfurtrifluoride and acid hydrolysis (P. K. G. Hodgson and W. Korytnyk, unpublished results). This material was then purified by descending paper chromatography on Whatman No. 1 paper (Whatman, Inc., Clifton, NJ) using ethanol–1 M ammonium acetate (85:15) as the solvent system. This purified material, which had a specific activity of 0.95 Ci/mmole, was stored at −20° in 90% ethanol. Under these conditions [^{3}H]-6-Fgal was found to be stable.

Treatment of L1210 leukemic cells in vitro *and* in vivo. Cultures of L1210 leukemic cells were maintained in logarithmic growth in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, NY) and a 16 mM HEPES, 8 mM MOPS buffer, pH 7.35 (Sigma Chemical Co., St. Louis, MO), at 37° in a humidified atmosphere containing 5% CO_2. In glucose-free RPMI 1640 medium, galactose but not 6-F-galactose could support cell growth. This L1210 cell line was free of contamination by mycoplasma as determined by direct culture methods in nutrient broth or agar [18], uridine phosphorylase assay [19], and microscopic visualization of extranuclear DNA by fluorescent staining techniques [20] using bisbenzimide fluorochrome 33258 stain (Hoechst Pharmaceuticals, Somerville, NJ). The L1210 leukemia ascites line was maintained *in vivo* by serial passage in syngeneic DBA/2 (HaDD) hosts. The ascites cells were obtained from mice inoculated with 1.0×10^6 viable cells on day 0 and treated on days 1–3 by continuous i.v. infusion [21] with various concentrations of gal (Sigma Chemical Co.) or 6-Fgal diluted in 0.9 NaCl, 0.1% sodium bicarbonate, pH 7.35. The mice were killed, and the ascites cells were removed, washed in PBS, and assayed as described. Numbers of cells were assessed with a model Z_F Coulter particle counter (Coulter Electronics, Inc., Hialeah, FL).

Incorporation studies. Determination of radiolabeled precursor incorporation was made in L1210 cells grown *in vitro* under the conditions described above with the addition of penicillin (50 units/ml) and streptomycin (50 μg/ml). Aliquots (2 ml) of L1210 cells (10^6 cells/ml) were incubated for 4 hr in the presence of 13 μCi of [6-^{3}H]-D-galactose (sp. act. 2.25 Ci/mole, New England Nuclear) or [^{3}H]-6-Fgal, and 1 mM unlabeled sugar. Other incorporation studies involved unlabeled 6-Fgal or gal and 2 μCi [^{3}H]-6-Fgal, 5 μCi [2-^{3}H]-D-mannose (sp. act. 12 Ci/mmole, Amersham Corp., Arlington Heights, IL), 10 μCi [6-^{3}H]-D-glucosamine (sp. act. 34 Ci/mmole, Amersham) or 1 μCi [3,4,5-^{3}H]-L-leucine (sp. act. 132 Ci/mmole, New England Nuclear) for 24 hr. In all experiments, the incorporation of labeled precursors into acid-insoluble material of control cells was linear throughout the incubation period. Incubations were terminated by washes with cold RPMI 1640 medium followed by precipitation with 1 ml of cold 10% (w/v) trichloroacetic acid (TCA). Nonenzymatic incorporation [22] of tritium into heat-inactivated fetal calf serum or boiled L1210 cells (10 min, 100°) was greater for [^{3}H]-gal than for [^{3}H]-6-Fgal using similar incubation conditions. This activity is presumably included in the results reported herein but represents only a fraction of the total incorporation measured. Aliquots of the resultant supernatant fractions were recovered (acid-soluble material), and the precipitates were re-washed in 10% TCA, dissolved in 1 N NaOH, and analyzed for protein by the method of Lowry *et al.* [23] with bovine serum albumin used as a standard. The radioactivity of the remaining sample was assessed by neutralization with 1 N HCl and counting in 3a70 complete scintillation mixture (Research Products International, Elk Grove Village, IL) with a Beckman model LS-100C liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA). Data were expressed as dpm/mg protein or percents of control and represented mean values and standard deviations of two or more experiments with four or more replicates per determination.

Electron microscopic autoradiography. L1210 cells were labeled with 50 μCi of [^{3}H]-gal or [^{3}H]-6-Fgal for 1 hr at 37° in RPMI 1640 medium without serum. The cells were thoroughly washed with cold RPMI 1640 medium, fixed for 2 hr in 3% phosphate-buffered glutaraldehyde (pH 7.0), post-fixed for 2 hr in 1% buffered osmium tetroxide, dehydrated in a graded alcohol series, and embedded in Epon-araldite. Ultramicrotome sections (100 nm thick) were mounted on collodionized slides, stained with 2% uranyl acetate, carbon-coated and overlaid with a monolayer of Kodak NTE emulsion (Kodak, Rochester, NY). The preparations were exposed for 30 days, developed in Dektol for 2 min at 24°, and photographed with a Siemens Elmiskop 101 electron microscope [14, 15].

Ectosialyltransferase assays. The assay procedures for L1210 cell surface sialyltransferase were similar to those described previously [14, 15, 24]. L1210 cells either from tissue cultures exposed for 48 hr to 5 mM gal, 3 mM 6-Fgal or 5 mM 6-Fgal, or from mice that were infused for 72 hr with gal or 6-Fgal ($2000\ mg \cdot kg^{-1} \cdot day^{-1}$) were collected and washed in serum-free RPMI 1640 and resuspended to 3.5×10^7 cells/ml. Aliquots of 0.5 ml were then incubated in the presence or absence of 0.025 units of *Vibrio cholerae* neuraminidase (EC 3.2.1.18. Calbiochem Behring, La Jolla, CA) for 15 min at 37°, washed several times with cold medium, and then incubated with 0.75 μCi of CMP-[^{14}C]-*N*-acetylneuraminic acid (CMP-[^{14}C]NANA, 259 mCi/mmole, Amersham Corp.) for 40 min at 37° in a shaking water bath. The final concentration of substrate was 0.56 mM. The reaction was terminated by the addition of 2 ml of cold 1% (w/v) phosphotungstic acid (PTA), and the precipitate was washed twice with 10% TCA and extracted with 2 ml of 95% ethanol–ethyl ether (1:1, v/v). The acid-insoluble material was dissolved and assayed for protein, and its radioactivity was measured as described previously. Results were expressed as pmoles NANA incorporated into neuraminidase generated acceptors per mg protein per hr. The

incorporation of [^{14}C]NANA from CMP-[^{14}C]NANA, by neuraminidase-treated cells, was essentially linear during the incubation period with no lag phase, and these results are similar to those reported earlier [24] and by others [25]. Incorporation of [^{14}C]NANA into the same number of L1210 cells, not preincubated with neuraminidase, was less than 5% of the incorporation by neuraminidase-treated cells. Previous work has shown that the incorporated [^{14}C]NANA by neuraminidase-treated L1210 cells is localized to the cell surface [14, 15] and is covalently attached by a cell surface sialyltransferase.

Sialyltransferase activity of cell homogenates. The assay for sialyltransferase in homogenates of L1210 cells was similar to that described for human serum [26, 27]. Cells were washed in RPMI 1640 medium, resuspended to 10^7 cells/ml, and disrupted by two cycles of nitrogen decavitation in a Parr bomb (Parr Instruments Co., Moline, IL). Several 50 μl aliquots of the homogenate were preincubated for 15 min at 37° in a mixture of 5 mM $MgCl_2$ and 0.1% Triton X-100 in 0.1 M cacodylate buffer, pH 6.5, in a total volume of 0.1 ml. Following preincubation, 0.3 mg fetuin (Grand Island Biological Co.), which had been desialylated by the method of Spiro [28], and 0.5 μCi CMP-[^{14}C]NANA were added and incubated for 1 hr at 37° in a shaking water bath. Incorporated material was precipitated with 1% PTA and 10% TCA, and the samples were processed as described above. Data were corrected for NANA transferred to endogenous substrates and were expressed as pmoles of NANA transferred to exogenously added desialylated fetuin per mg protein per hr.

Membrane 5′-nucleotidase activity. Plasma membrane-enriched subcellular fractions were obtained by the method of Hourani *et al.* [29] and were found to be enriched up to 20-fold in 5′-nucleotidase activity (EC 3.1.3.5). The membrane fraction was washed twice and resuspended in a 0.1 M Tris buffer containing 0.1% Triton X-100. An aliquot of this crude enzyme preparation was then added to a reaction mix of 0.1 M Tris buffer, pH 8.5, 10 mM $MgCl_2$ and either 6 mM 5′-AMP or 6 mM 3′-AMP in a total volume of 1 ml and incubated for 1 hr at 37°. The reaction was terminated by the addition of 1.0 ml of 10% TCA, and aliquots of the supernatant fractions were assayed for PO_4^{2-} release by the ascorbate method of Chen *et al.* [30] using K_3PO_4 as a standard. Data, corrected for endogenous PO_4^{2-} (boiled controls) and 3′-PO_4^{2-} released, were expressed as μmoles of 5′-PO_4^{2-} released per mg protein per hr.

Sialic acid released by acid hydrolysis or neuraminidase. 6-Fgal-treated or control cells were washed in cold RPMI 1640, suspended to 1×10^7 cells/ml and disrupted by nitrogen decavitation, and centrifuged at 103,000 *g* for 45 min in a Beckman model L5-50 preparative ultracentrifuge (Beckman Instruments, Inc.). Half of the pellet was hydrolyzed with 2 ml of 1 N HCl at 80° for 1 hr, and the soluble material was assayed for sialic acids by the thiobarbituric acid (TBA) method of Aminoff [31]. The other half of the pellet was extracted with chloroform–methanol (2:1, v/v) for 30 min, and the extract was assayed for sialic acid content by the method of Svennerholm [32]. In three experiments, the chloroform-soluble sialic acid was 5% or less of the total acid hydrolyzed sialic acid.

Cell surface sialic acids released with neuraminidase were quantitated in control cells or cells exposed to 6-Fgal *in vitro* or treated with the analogue *in vivo* by continuous infusion. Following 6-Fgal treatment, cells were washed three times in PBS, suspended at 1×10^8 cells/ml in 0.05 ml of acetate buffer (0.05 M sodium acetate, 0.15 M NaCl and 8 mM $CaCl_2$ at pH 5.5), and incubated with 0.2 units of *V. cholerae* neuraminidase for 30 min at 37° in a shaking water bath. The reaction was terminated by immersion of the tubes in ice water, and the mixture was centrifuged at 1200 *g* at 4°. The supernatant fraction was extracted with 10% cold TCA and centrifuged. This final supernatant fraction, which contained the acid-soluble, neuraminidase-released sialic acid, was assayed by the method of Aminoff [31]. Aliquots of cells, to which an equal amount of heat-denatured neuraminidase was added, were incubated in parallel and served as background in the spectrophotometric assay.

Analysis of intracellular ^{3}H-sugar metabolites. L1210 leukemia cells were incubated *in vitro* for 4 hr with 0.11 Ci/mole of either 1 mM [^{3}H]-gal or [^{3}H]-6-Fgal. The cells were washed thoroughly, and 1×10^7 cells were extracted for 10 min at 4° with 0.6% perchloric acid (PCA) in 0.1 N ammonium formate, pH 4.4. The extracts were then neutralized with 0.1 N KOH and centrifuged, and the supernatant fractions were chromatographed in a reverse phase, ion-pair high pressure system with an Altex Ultrasphere column (250 × 4.6 mm i.d.) and a Whatman ODS guard column (Whatman Inc.) at 2000 psi. Fractions of 0.50 ml were collected with an LKB model 2112 Redirac fraction collector (LKB Instrument, Inc., Hicksville, NY), and radioactivity was determined as described above. [^{3}H]-6-Fgal, [^{3}H]-gal and UDP-D-[1-^{3}H]-galactose (New England Nuclear) were used as standards.

In another experiment, L1210 leukemia cells incubated *in vitro* for 4 hr with either [^{3}H]-gal or [^{3}H]-6-Fgal were extracted and washed thoroughly with 10% TCA. The resultant cellular pellets were hydrolyzed for 18 hr with 1.0 N HCl and neutralized, and the resultant hydrolysates were analyzed on orange ribbon paper following descending chromatography for 18 hr using butanol, pyridine and 0.1 N HCl (5:3:2) as the solvent system. Some radioactivity remained at the origin but the majority co-chromatographed with either authentic [^{3}H]-gal or the [^{3}H]-6-F-gal respectively.

RESULTS

6-Fgal and gal at 5 mM specifically reduced the incorporation of [^{3}H]-gal while it had little effect on the incorporation of [^{3}H]leucine and no effect on the incorporation of labeled D-mannose or D-glucosamine (Fig. 1). For both [^{3}H]-gal and 6-Fgal, incorporation was localized by electron microscope autoradiography as silver grains concentrated mainly over the Golgi region of L1210 cells after 1 hr of labelling with only a few of the grains associated with the

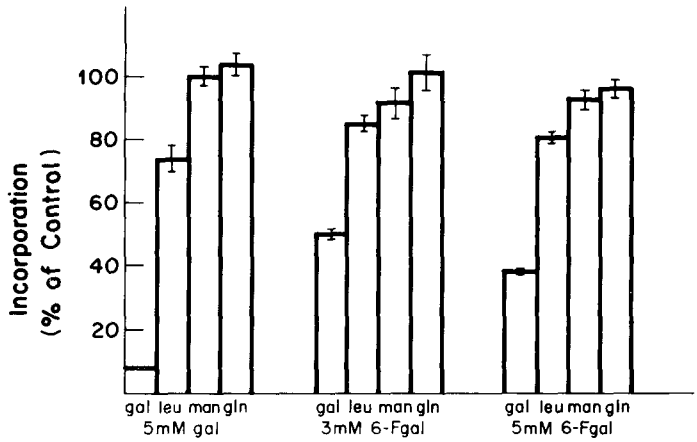

Fig. 1. Effects of 5 mM D-galactose or 3 mM and 5 mM 6-Fgal on the incorporation of tritiated D-galactose (gal), L-leucine (leu), D-mannose (man) and D-glucosamine (gln) into L1210 cell acid-insoluble materials during a 24-hr incubation *in vitro*. Experimental means (cpm/mg protein) and standard deviations are expressed as percent control (without added hexose) values, which exceeded 40,000 cpm/mg protein for each determination.

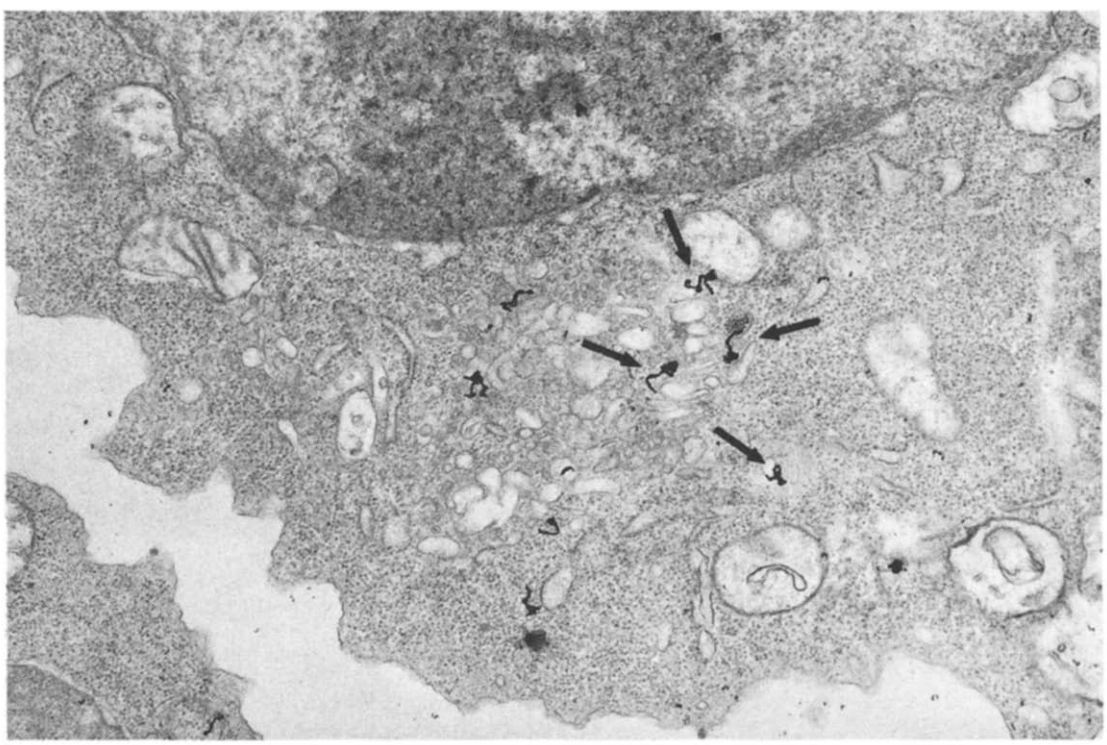

Fig. 2. Electron microscope autoradiograph of the incorporation and localization of [^{3}H]-6-Fgal at the Golgi region of an L1210 cell labeled for 1 hr. Arrows indicate silver grains in Golgi regions. The silver grain distribution was similar for cells labeled with [^{3}H]-gal under similar conditions. (Magnification: × 10,320.)

Table 1. L1210 cell acid-insoluble and -soluble pools of [^{3}H]-gal and [^{3}H]-6-Fgal in the absence or presence of L-fucose*

L-Fucose (mM)	[^{3}H]-D-Galactose (dpm/mg protein × 10^{-3})			[^{3}H]-6-F-D-Galactose (dpm/mg protein × 10^{-3})		
	Acid-insoluble	Acid-soluble	Insoluble/Soluble	Acid-insoluble	Acid-soluble	Insoluble/Soluble
0 (Control)	2.1 ± 0.10	103.5 ± 7.5	0.020	14.6 ± 0.74	122.5 ± 6.9	0.12
5.0	1.8 ± 0.06	90.1 ± 2.7	0.020	14.9 ± 0.34	106.3 ± 9.9	0.15

* L1210 cells (10^6 cells/ml) were incubated for 4 hr with either 1 mM [^{3}H]-gal or [^{3}H]-6-Fgal (6.5 μCi/ml for each) in 2 ml of RPMI 1640 containing 10% heat-inactivated fetal calf serum. Radioactivity was measured as described in Materials and Methods. Five determinations were made at each concentration of fucose, and the mean ± S.D. is listed.

nucleus or plasma membrane (Fig. 2). Only radioactive sugar incorporated into glycoconjugate, and not that present in the cell as free sugar or nucleotide sugar, is retained after processing of tissue for electron microscopy [14].

The analogous nature of 6-Fgal to gal was further demonstrated in studies which compared the incorporation of [^{3}H]-gal and [^{3}H]-6-Fgal. When L1210 leukemic cells were incubated with [^{3}H]-gal or [^{3}H]-6-Fgal at the same concentration and specific activity for 4 hr, more acid-soluble and acid-insoluble 6-Fgal was accumulated intracellularly and the ratio of acid-insoluble : acid-soluble counts was 6- to 7-fold greater for 6-Fgal than for gal (Table 1). The possibility that 6-Fgal was incorporated to a greater extent because of some structural similarity to L-fucose was dismissed since a 5-fold excess concentration of L-fucose did not affect the amounts of acid-soluble or acid-insoluble counts of either 6-Fgal or gal (Table 1). The amount of [^{3}H]-6-Fgal incorporated nonenzymatically was low and less than that of [^{3}H]-gal and, therefore, could also not account for this difference.

The PCA-soluble metabolites of [^{3}H]-gal and [^{3}H]-6-Fgal from L1210 cells, incubated under similar conditions as in the studies summarized in Table 1, were analyzed by reverse phase ion-pair HPLC and gave the chromatographic profiles shown in Fig. 3. In each of three separate experiments, there was some variance in the relative magnitudes of peaks A and B (sugar and sugar 1-PO_4) for both 6-Fgal and gal, but the amount of radioactivity which co-chromatographed with UDP-[1-^{3}H]-D-galactose (peak C) was similar for both sugars in all determinations (Fig. 3).

Treatment of L1210 cells with 6-Fgal, both *in vitro* and *in vivo*, resulted in a dose-dependent decrease in the activity of ectosialyltransferase (Fig. 4). This effect did not appear to be caused by a non-specific perturbation of the plasma membrane since the activity of membrane 5′-nucleotidase was unchanged in 6-Fgal-treated cells (Table 2). The decrease in ectosialyltransferase activity was dependent on the length of exposure of the tumor cells to the analogue

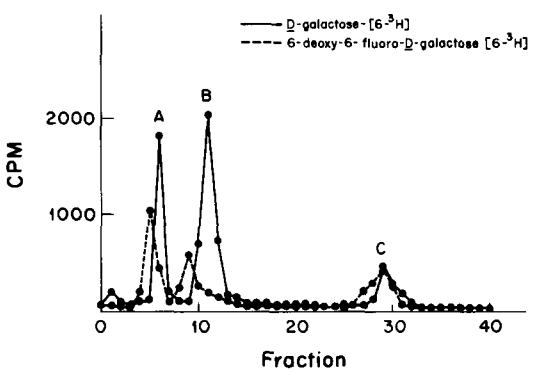

Fig. 3. Chromatographic profile of [^{3}H]-D-gal (——) and [^{3}H]-6-Fgal (– – – –) acid-insoluble metabolites following reverse phase, ion-pair HPLC. Peaks A, B and C co-eluted with tritiated galactose, galactose-1-PO_4 and UDP-galactose standards respectively.

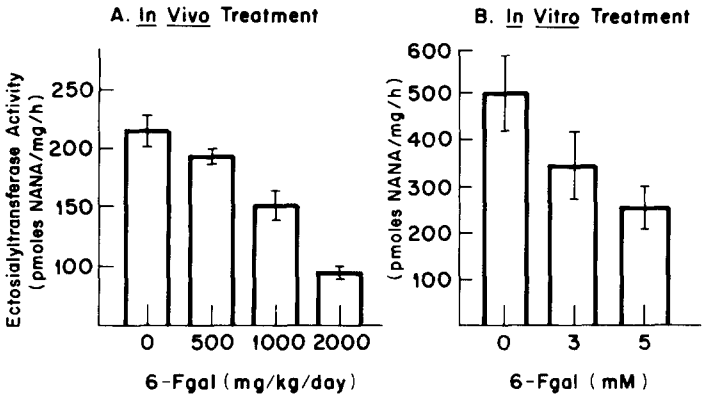

Fig. 4. Ectosialyltransferase activity in L1210 cells following (A) treatment of mice by i.v. infusion for 3 days with 2000 mg·kg^{-1}·day^{-1} D-galactose (0 representing the saline control) or the given doses of 6-Fgal, and (B) treatment of L1210 cultured cells with 5 mM D-galactose (control) or 3 and 5 mM 6-Fgal for 48 hr.

and, if 6-Fgal was added directly to the enzyme assay mixture of untreated cells, there was no effect on the amount of NANA transferred (data not shown). The decrease in sialyltransferase activity appeared to be specific for the cell surface enzyme since the total activity of sialyltransferases in homogenates of cells treated under the same conditions *in vitro* was unchanged (Table 2). The data demonstrated that the capacity of the 6-Fgal-treated cells to transfer NANA to exogenous acceptors (desialylated fetuin) was equal to that of control cells.

Finally, the amount of sialic acid which could be acid hydrolyzed from homogenates of cells treated with 6-Fgal did not differ significantly from that of control cells (Table 2). Similarly, the amount of NANA which was removable from the surface of intact cells with neuraminidase was not decreased by exposure to 6-Fgal for 48 hr *in vitro* (Table 2). It was apparent, however, that the absence of an overall effect on sialic acid metabolism could not be accounted for by insufficient activation or incorporation of the analogue in L1210 cells *in vitro* (Fig. 3 and Table 1). Moreover, there did not appear to be a selective removal of 6-Fgal containing acceptors from the cell surface. The rate of appearance of modified acceptors on the cell surface, as measured by the decrease in ectosialyltransferase activity following exposure of L1210 cells to 5 mM 6-Fgal (Fig. 5A), was equivalent in the first 24 hr of exposure to 6-Fgal to the rate of disappearance of modified acceptors (or increase in ectosialyltransferase activity) within the first 24 hr following removal of 6-Fgal from the culture medium (Fig. 5B).

DISCUSSION

In these studies, 6-Fgal was demonstrated to be a functional analogue of gal on the basis of its specific inhibition of [^{3}H]-gal incorporation (Fig. 1), a measurement which relates to competition between the fluorinated analogue and its parent sugar for uptake, activation to nucleotide-sugar and incorporation [33]. Earlier studies by Barnett *et al.* [34] had demonstrated that 6-Fgal, but not 6-deoxy-D-

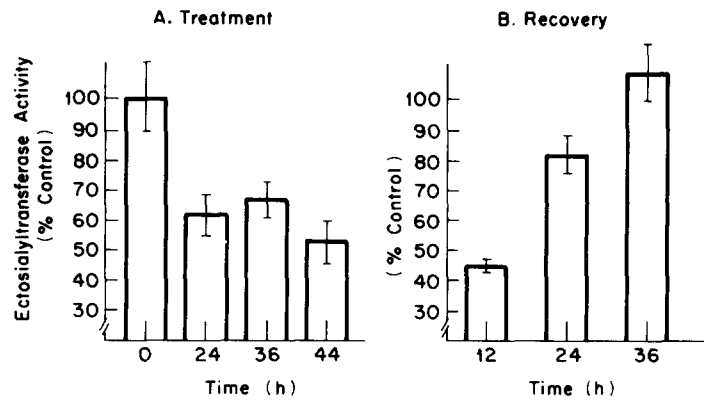

Fig. 5. Onset (A) and recovery (B) kinetics of ectosialyltransferase activity in L1210 cells in the presence of 5 mM 6-Fgal (A) and following its removal from the medium (B). Data are expressed as percents of 5 mM D-gal control values at each time point.

Table 2. Demonstration of the lack of effect of 6-Fgal on L1210 cell 5'-nucleotidase activity and sialic acid metabolism *in vitro**

	Membrane 5'-nucleotidase activity (μmoles phosphate/mg protein/hr	Cell homogenate sialyltransferase activity (pmoles NANA/mg protein/hr)	Acid-hydrolyzed sialic acid (nmoles TBA positive material/mg protein)	Neuraminidase-released sialic acid (nmoles TBA positive material/mg protein)
5 mM D-Galactose	3.93 ± 0.38	24.5 ± 2.8	1.99 ± 0.32	0.84 ± 0.02
3 mM 6-Fgal	3.69 ± 0.39	22.5 ± 2.3	2.24 ± 0.5	0.88 ± 0.04
5 mM 6-Fgal	3.82 ± 0.33	24.1 ± 3.2	1.78 ± 0.26	0.84 ± 0.02

* L1210 cells (10^5 cells/ml) were suspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum and were incubated for 48 hr with either 5 mM gal, 3 mM 6-Fgal or 5 mM 6-Fgal. Cells were harvested and used for the various assays listed above. All determinations were performed in quadruplicate on at least three separate occasions. All data are listed as means ± S.D. Control values (*no additions* of gal or 6-Fgal) were not significantly different from any of the values listed above (data not shown).

galactose, was transported in the gut nearly as well as gal, and suggested that this was due to the ability of the fluorine at the C-6 position to participate in hydrogen bonding with the transport protein. Along similar lines, Kent and Wright [35] and Thomas *et al.* [36] had shown that 6-Fgal was a suitable substrate of yeast galactokinase, and Kent and Wright [35] also demonstrated that 6-Fgal was reduced three times faster than gal in a rabbit lens capsule system. Under conditions which yielded rather similar amounts of intracellular, acid-soluble metabolites of either gal or 6-Fgal, [^{3}H]-6-Fgal was incorporated into L1210 leukemic cell acid-insoluble material at a 6- or 7-fold greater extent than was [^{3}H]-gal (Table 1). The apparently preferential incorporation of the sugar analogue over that of the parent sugar was unexpected, but a similar result has also been reported by Buchsell *et al.* [37] for 2-deoxy-D-galactose, another galactose analogue. The findings reported herein for 6-Fgal do not seem to be based on catabolism, nonenzymatic incorporation, or on an unequal formation of UDP-6-Fgal or UDP-gal. Further, the presence of L-fucose, did not affect the amounts of acid-soluble or acid-insoluble tritiated sugar. Similarly, preincubation of L1210 cells with 6-Fgal for 48 hr did not alter the incorporation of [^{3}H]-L-fucose (data not shown) which is often attached to oligosaccharides by an α (1→2) glycosidic linkage to galactose [38]. All of these studies demonstrated quite conclusively that 6-Fgal is a biologically active analogue of galactose which can serve as a substrate in a number of different enzymatic pathways.

The intracellular incorporation of 6-Fgal into glycoprotein was localized by electron microscope autoradiography and was found to take place, like [^{3}H]-gal, in the Golgi region of L1210 cells following an exposure period of 1 hr. In another study using electron energy loss spectroscopy, fluorine was detectable in the Golgi region of sections of L1210 cells following incubation of cells with 6-Fgal [39]. The specific localization of the analogue at the level of the Golgi may reflect the lack of catabolism of the labeled analogue *in situ*. Additional studies have demonstrated that, in glucose-free media, 6-Fgal is not a suitable carbon source in the maintenance of L1210 cell growth. In the presence of high amounts of glucose, as in RPMI 1640 medium, both [^{3}H]-gal and [^{3}H]-6-Fgal become phosphorylated, form nucleotide-sugars (Fig. 3), and become incorporated into acid-insoluble material. The incorporated label can be recovered by acid hydrolysis and was found to contain predominantly authentic [^{3}H]-gal or [^{3}H]-6-Fgal. Taken together, these studies indicate that the sugar analogue is predominantly intact following its incorporation into glycoconjugate.

At non-growth inhibitory concentrations *in vitro* or at doses which did not affect host survival *in vivo*, exposure of L1210 cells to 6-Fgal resulted in dose- and time-dependent decreases in the enzymatic transfer of NANA at the cell surface. Ectosialyltransferase activity was wholly dependent on the number of neuraminidase-treated cells, was linear for the duration of the assay period [24], and endogenous activity, which may be related to the uptake of free [^{14}C]NANA and its incorporation into

intracellular acceptors for sialyltransferase, never exceeded 5% of the total activity measured. The measurement of ectosialyltransferase activity has been demonstrated by this laboratory [14, 15, 24] and others [25] to reflect the direct transfer of NANA via the CMP-NANA substrate, specifically at the cell surface, and not by the intracellular incorporation of free NANA. The specificity of the effect induced by 6-Fgal on ectosialyltransferase activity was demonstrated by the lack of significant changes in the activities of total cellular sialyltransferase (to exogenous acceptors) or of plasma membrane 5′-nucleotidase. Thus, the decrease in the activity of ectosialyltransferase is postulated to be due to a decrease in suitable membrane-bound acceptors by virtue of the incorporation of a C-6 halogen-substituted galactose, which would preclude the α (2→6) glycosidic attachment of NANA. This effect, which resulted in a decrease in the transfer of NANA at the cell surface of up to 50% of control levels *in vitro* and *in vivo*, however, did not manifest itself as a decrease in the total cellular sialic acid which could be acid-hydrolyzed from cell homogenates, nor did it result in a decrease in the amount of NANA released from cell surface with neuraminidase. One possible reason for this result might be the structures of the oligosaccharides that are indigenous to L1210 cells. In mammalian glycoproteins, the most common linkage types for sialic acid to galactose are the α (2→6) and α (2→3) glycosidic types [40], but there is little information about which linkage type, if any, predominates *in situ*. As demonstrated in Fig. 6, a given glycoprotein may be heterogeneous in its NANA linkage types, as is the case for an oligosaccharide of fetuin which has one NANA residue linked α (2→6) and two linked α (2→3) to galactose [41]. Conversely, human lactoferrin contains a single α (2→6) linked NANA [42] and human MN antigen contains one α (2→3) linked sialic acid residue [43]. Cells which had significantly more of either the α (2→6) or the α (2→3) linkage type may inherently be more sensitive or resistant to the effects of 6-Fgal with regard to the attachment of sialic acid to their glycoproteins.

Applying this premise to the findings with 6-Fgal in L1210 cells reported herein, one could infer that the oligosaccharides of the cell surface acceptors for ectosialytransferase were similar to those of fetuin, and that exposure of L1210 cells to 6-Fgal may result in a block of the attachment of the α (2→6) linked, but not the α (2→3), bonds of the NANA residues normally attached to galactose. Further, a predominance of an α (2→3) glycosidic linkage between sialic acid and galactose in a majority of the other oligosaccharides of L1210 cell glycoproteins and glycolipids would have resulted in the observed lack of effect on total sialic acids. Alternatively, one could also infer that ectosialyltransferase is specific for α (2→6) linkage to galactose and is thus very susceptible to the incorporation of 6-Fgal into membrane glycoproteins.

Nonetheless, it has become apparent from our studies and others that sugar analogues may be used as specific probes of glycoconjugate biosynthesis, structure, and metabolism. 6-F-Galactose was found to be a biologically active analogue of galactose. L1210 leukemic cells were capable of activating it to form nucleotide-sugar and incorporating it, intact, in the Golgi apparatus. At a later time, presumably due to the insertion of analogue containing oligosaccharides into the cell surface, a dose-dependent decrease in ectosialyltransferase was measured. This effect was specific for sialyltransferase and presumably was the result of the formation of structurally

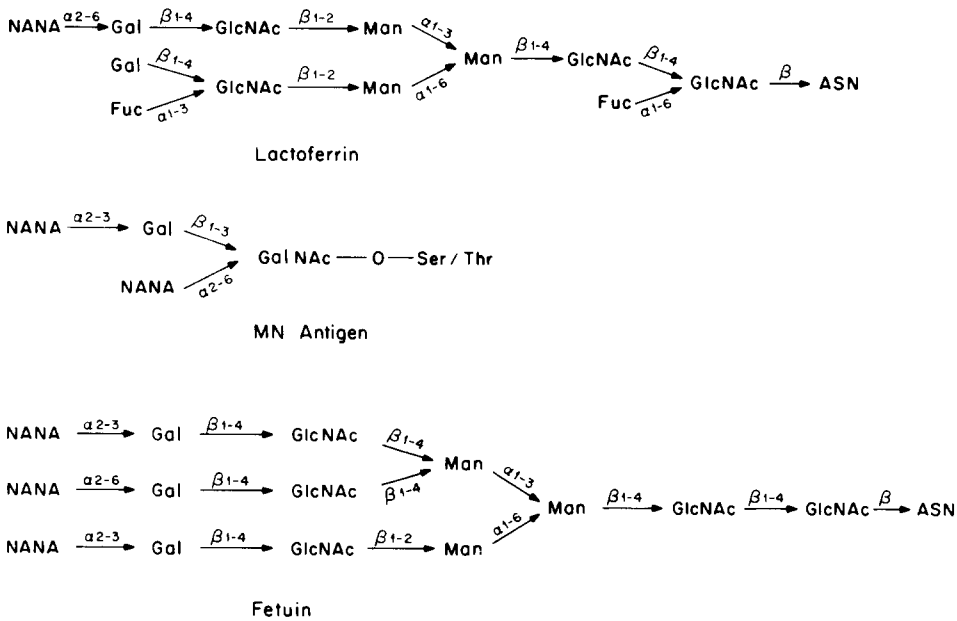

Fig. 6. Structural representation of three common sialoglycoproteins demonstrating distinct differences in the number of α (2→3) and α (2→6) linkages between NANA and galactose.

modified oligosaccharide acceptors unable to serve as suitable substrates for the plasma membrane located sialyltransferase. Greater knowledge of the chemistry of cellular glycoconjugates should provide a more rational basis for the design of biochemically and therapeutically useful sugar analogues.

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