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EFFECTS OF TUNICAMYCIN, *N*-METHYL-1-DEOXYNOJIRIMYCIN, AND MANNO-1-DEOXYNOJIRIMYCIN ON THE BIOSYNTHESIS OF LACTOSAMINOGLYCANS IN F9 TERATOCARCINOMA CELLS*

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ABSTRACT

F9 teratocarcinoma cells were incubated with *D*-[2-^{3}H]mannose or *D*-[6-^{3}H]galactose, and the labeled glycopeptides obtained after exhaustive digestion by pronase were fractionated on Bio-Gel P-6 before and after treatment by endo-β-*N*-acetylglucosaminidase H. Tunicamycin almost completely inhibited the synthesis of lactosaminoglycans found in excluded glycopeptides of large molecular weight. Manno-1-deoxynojirimycin greatly inhibited the incorporation of labeled mannose into both lactosaminoglycan and complex oligosaccharides, while it greatly increased that into $Man_8GlcNAc$ and $Man_9GlcNAc$ oligosaccharides. In contrast, *N*-methyl-1-deoxynojirimycin only partially inhibited the incorporation into lactosaminoglycan and complex oligosaccharides, and caused the accumulation of $Glc_3Man_{7-9}GlcNAc$ oligosaccharides. These results demonstrate that, in these cells, lactosaminoglycans are *N*-linked, and suggest that there is transfer of both glucosylated and nonglucosylated oligosaccharides from lipid to protein.

INTRODUCTION

One of the characteristics of the cell surface of mouse embryonal carcinoma cells (EC cells) and mouse preimplantation embryos is the presence of fucosylated oligosaccharides of large molecular weight containing[1,2] the repeating disaccharide, β-Gal-(1→4)-β-GlcNAc-(1→3). The synthesis of this highly branched type of lactosaminoglycan is greatly decreased during differentiation of EC cells and during embryonic development. Several developmentally regulated antigens[3,4] and lectin receptors[3] are located on these structures, which have been suggested to play a role in EC cell adhesion[5].

Lactosaminoglycans are also found on other cells, such as erythrocytes[6], granulocytes[7], .CHO cells[8], and in some tumor cells where they have been implicated in the metastatic properties of the cells[9]. In most cases, the lactosaminoglycans are *N*-linked, although they may also occur as *O*-linked oligosaccharides in

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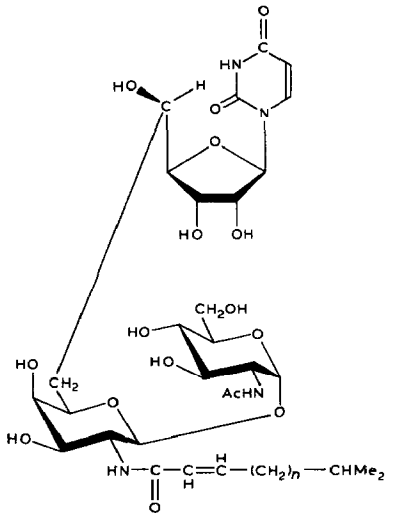
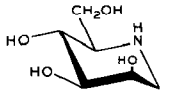


Tunicamycin (1), n = 8,9,10, or 11



Manno-1-deoxynojirimycin

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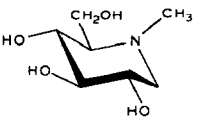

N-Methyl-1-deoxynojirimycin

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mucins. The structure of lactosaminoglycans of EC cells has been extensively investigated, but the exact nature of the glycopeptide linkage has not been conclusively established. Although specific glycosyltransferases responsible for the synthesis of the repeating disaccharide units have been characterized[10–12], little is known about the initial stages of the processing pathway that leads to their biosynthesis. In the present work we have examined the effect of specific inhibitors of *N*-linked glycosylation (tunicamycin, **1**) and of *N*-linked oligosaccharide processing [(manno-1-deoxynojirimycin, **2**) and *N*-methyl-1-deoxynojirimycin (**3**)] on the biosynthesis of lactosaminoglycans in F9 teratocarcinoma cells.

RESULTS AND DISCUSSION

When F9 teratocarcinoma cells were incubated for 24 h with D-[2-^{3}H]-mannose and the glycopeptides obtained by exhaustive pronase digestion were fractionated on a column of Bio-Gel P-6, two labeled fractions were obtained: one glycopeptide fraction was excluded from the gel and eluted in the void volume of the column (Fraction I), whereas the major labeled glycopeptide fraction was included with a K_{av} of ~0.45 (Fig. IA–D). When the cells were incubated with D-[6-^{3}H]galactose under the same conditions, ~60% of the radioactivity incorporated into glycopeptides was found in the excluded fraction (data not shown). From the studies of Muramatsu *et al.*[1,2], the excluded glycopeptide fraction of large molecular weight contains the lactosaminoglycans characteristic of F9 teratocarcinoma cells.

The included glycopeptides were further resolved into two fractions by treatment with endo-β-*N*-acetylglucosaminidase H (endo H) (Fig. IE–G). A major part of the labeled glycopeptides (K_{av} of ~0.45, Fig. 1A–D) were transformed into

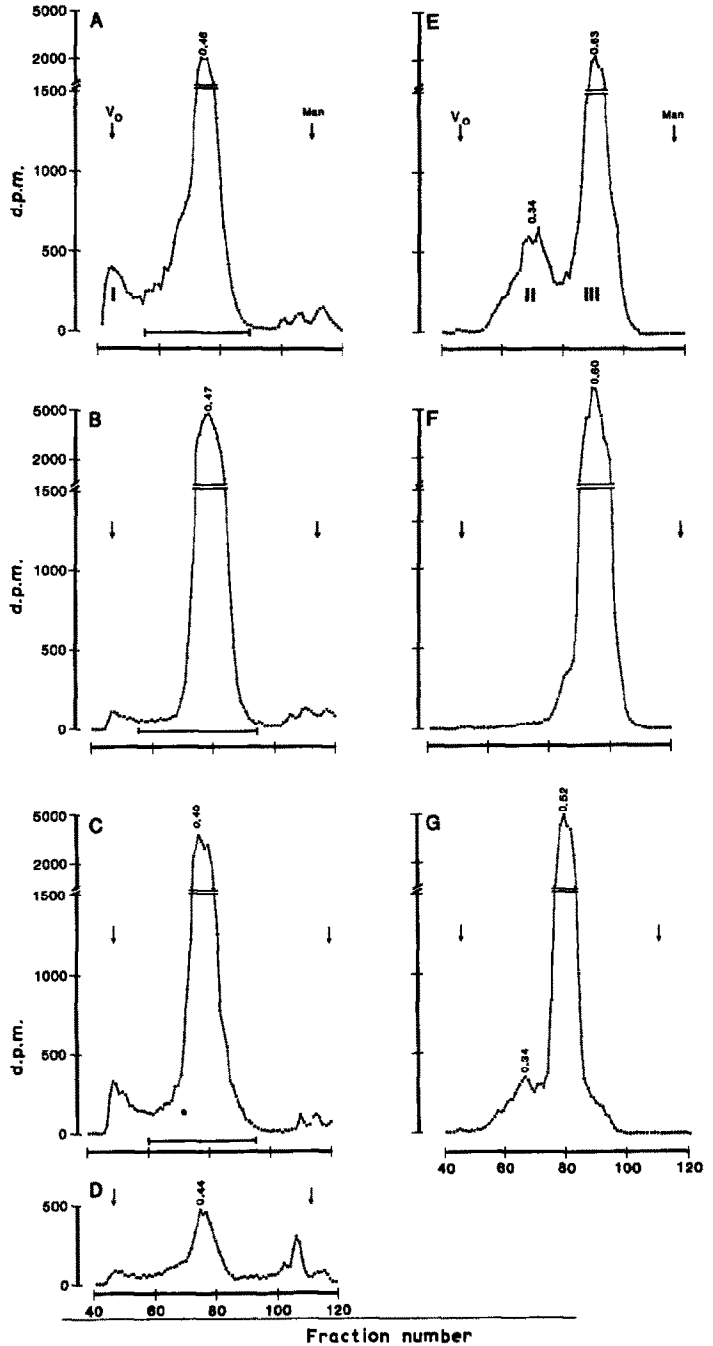

Fig. 1. Effect of inhibitors on glycopeptides from F9 cells incubated with D-[2-^{3}H]mannose. Labeled glycopeptides obtained by exhaustive pronase digestion were first fractionated on a column of Bio-Gel P-6 (A–D). The glycopeptides in fractions indicated by the bar were then incubated with endo H as described in the Experimental section and the products were chromatographed on the same column of Bio-Gel P-6 (E–G). A and E, control cells; B and F, cells treated with mM **2**; C and G, cells treated with 2mM **3**; D, cells treated with 1 μg/mL tunicamycin (**1**). The aliquot from the column in D was 2.5 times greater than that counted in A–C. Values of K_{av} are indicated above the peaks.

TABLE I

EFFECT OF THE INHIBITORS ON THE GLYCOPEPTIDES AND OLIGOSACCHARIDES OF F9 CELLS

Inhibitor	*Concentration*	*Fraction[a] I*	*Fraction[a] II* d.p.m. (%)	*Fraction[a] III*
None		101,870 (100)	224,500 (100)	562,300 (100)
2	mM	24,290 (24)	17,400 (8)	1,346,300 (239)
3	2mM	65,700 (65)	101,600 (45)	838,400 (149)
1	1 μg/mL	10,970 (11)	97,770[b]	

[a]Fractions I, II, and III were obtained from the Bio-Gel P-6 columns of Fig. 1. [b]Sample was not treated with endo H.

TABLE II

PRODUCTS OF α-D-MANNOSIDASE TREATMENT OF OLIGOSACCHARIDES

Additions	*Concentration* (mM)	Glc_1[a]	Glc_2[a]	Glc_3[a]	*Mannose*[b]
		d.p.m. (%)			
None		250(3)	130(1)	100(1)	8,950(95)
2	1	210(1)	180(1)	370(2)	19,340(96)
3	2	270(3)	390(5)	3,540(40)	4,565(52)

The endo H-sensitive oligosaccharides obtained after Bio-Gel P-6 chromatography were exhaustively digested with jack bean α-D-mannosidase as described in Experimental and the products were fractionated by l.c.
[a]Glc_1, $Glc_1Man_4GlcNAc$; Glc_2, $Glc_2Man_4GlcNAc$; Glc_3, $Glc_3Man_4GlcNAc$. [b]Under these conditions, mannose and the β-Man-(1→4)-GlcNAc disaccharide are not separated.

species of lower molecular weight with a K_{av} of ~0.6, thereby indicating that these consisted primarily of endo H-sensitive, high-mannose oligosaccharides (Fraction III). The rest of the material (shoulder seen in Fig. 1A with a K_{av} of ~0.34) was unaffected by endo H treatment, and most probably contains *N*-linked complex oligosaccharides (Fraction II). In all experiments described, the medium contained unlabeled L-fucose, as it was observed that in its absence ~17% of the radioactivity in the total glycopeptides, and ~50% of the radioactivity in the lactosaminoglycan fraction, was recovered as labeled L-fucose. In the presence of 2mM unlabeled L-fucose in the medium, however, acid hydrolysis of the glycopeptides followed by paper chromatography demonstrated that all the radioactivity remained associated with mannose.

Tunicamycin (**1**), a known inhibitor of *N*-linked glycosylation, caused 90% inhibition of mannose incorporation into F9 cell glycoproteins at a very low concentration (1 μg/mL), whereas methionine incorporation into proteins was only decreased ~30%. Under these conditions, the incorporation of labeled mannose and galactose into the large-molecular-weight glycopeptides of Fraction I was inhibited ~90 and 80%, respectively. The fact that labeled mannose is incorporated as such into lactosaminoglycans and the nearly complete inhibition of both mannose

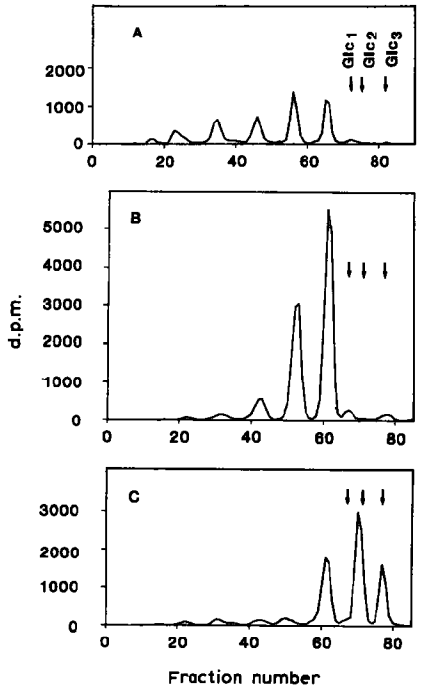

Fig. 2. L.c. of endo H-sensitive oligosaccharides. The labeled oligosaccharides released by endo H obtained from Bio-Gel P-6 (Fraction III, Fig. IE–G) were fractionated by l.c. as described under Experimental. A, control cells; B, cell treated with mM **2**; C, cells treated with 2mM **3**. The arrows indicate the elution position of [^{14}C]labeled standards: Glc_1, $Glc_1Man_9GlcNAc$; Glc_2, $Glc_2Man_9GlcNAc$; Glc_3, $Glc_3Man_9GlcNAc$.

and galactose incorporation observed with tunicamycin indicates that, in F9 cells, these oligosaccharides are primarily *N*-linked.

Incubation of the cells with **2**, which has been shown to inhibit Golgi α-D-mannosidase I involved in the processing of *N*-linked oligosaccharides[13], greatly inhibited the incorporation of labeled mannose into both the complex and lactosaminoglycan fractions, while greatly increasing the incorporation of mannose into the endo H-sensitive oligosaccharides (Fig. IB and F, Table I).

Incubation of the cells with **3**, an inhibitor of processing α-D-glucosidases, had similar effects on mannose incorporation into the various fractions, except that the inhibition of complex and lactosaminoglycan synthesis was much less pronounced (Fig. 1, C and G, Table I). The inhibition observed with **3** was similar when the cells were preincubated with the inhibitor for 24 h, and when the concentration of **3** was increased to as much as 8mM.

The endo H-sensitive, labeled oligosaccharides (Fraction III) were fractionated by l.c. in order to characterize the oligosaccharides formed under the various conditions. In control and **2**-treated cells, the labeled oligosaccharides were eluted earlier than the glucosylated oligosaccharide standards, and corresponded to $Man_{5-9}GlcNAc$ (Fig. 2 A, B). The presence of **2** caused a large increase in

labeled $Man_8GlcNAc$ (three-fold) and $Man_9GlcNAc$ (five-fold), a decrease in $Man_5GlcNAc$ and $Man_6GlcNAc$ (about 70%), and little change in $Man_7GlcNAc$. The absence of glucosylated oligosaccharides in both control and **2**-treated cells was confirmed by incubation with jack bean α-D-mannosidase, which released ~95% of the radioactivity in the mannose-containing region (Table II).

The l.c. profile of the endo H-sensitive oligosaccharides obtained from cells incubated with **3** was very different. About 60% of the labeled oligosaccharides coeluted with the glucosylated standards (Fig. 2C). Treatment of the endo H-sensitive oligosaccharides with jack bean α-D-mannosidase indicated that the three labeled oligosaccharide fractions contained three glucose residues, as only 52% of the radioactivity was released, and 40% of the radioactivity was recovered in $Glc_3Man_4GlcNAc$ (Table II). From our previous studies[14], these results indicate that the labeled oligosaccharides found in the presence of **3** (Fig. 2C) are $Glc_3Man_{7-9}GlcNAc$. Furthermore, these results demonstrate that the inhibition of processing glucosidases by **3** in F9 cells was similar to that previously observed in other cells[14,15].

The present results demonstrating that the incorporation of mannose into lactosaminoglycans of F9 cells is inhibited by tunicamycin (**1**) and by the inhibitors of processing glycosidases, **2** and **3**, support the idea that these structures are primarily found as *N*-linked oligosaccharides, and that their synthesis follows a processing pathway similar to that described for other *N*-linked oligosaccharides. In these cells, the extent of inhibition of formation of *N*-linked complex oligosaccharides observed with **2** was similar to that reported in other cells[16,17]. On the other hand, the extent of inhibition of complex oligosaccharide synthesis with **3** was not as pronounced in these cells as that observed in rat intestinal epithelial cells[14] in which 80% inhibition was obtained, and for influenza virus glycoproteins in chick embryo cells in which complete inhibition was achieved[15]. The reasons for this difference between F9 cells and the other cells are not clear. It may be that the processing glucosidases in F9 cells are less sensitive to **3**, and/or that there is significant transfer of non-glucosylated oligosaccharide from dolichyl oligosaccharide diphosphate to protein in these cells. As the endo H-sensitive oligosaccharides formed in the presence of **3** contained three glucose residues, the inhibition of glucosidases by **3** seems to be as efficient as that observed in the other cells, so that transfer from non-glucosylated species seems most likely. This pathway of biosynthesis has been described in trypanosomatids[18-20] and in some yeast mutants[21,22]. The present results suggest therefore that both glucosylated and non-glucosylated oligosaccharides may be transferred to protein in F9 teratocarcinoma cells.

EXPERIMENTAL

Materials. — The source of chemicals was described previously[23]. L-Fucose was purchased from Pfanstiehl Laboratories, Waukegan, IL; bacto-gelatin was

purchased from Difco Laboratories, Detroit, MI; tunicamycin (**1**) was from Sigma Chemical Co., St. Louis, MO. D-[2-^{3}H]Mannose (specific radioactivity 24–27 Ci/mmol) and D-[6-^{3}H]galactose (specific radioactivity, 26–35 Ci/mmol) were purchased from New England Nuclear, Boston, MA, and from Amersham, Arlington Heights, IL, respectively. Manno-1-deoxynojirimycin (**3**) was obtained from Dr. G. Kinast, Bayer AG, Wuppertal, West Germany and *N*-methyl-1-deoxynojirimycin (**2**) from Dr. Y. Aoyagi, Nippon Shinyaku Co. Ltd., Kyoto, Japan.

Cell culture. — F9 cells obtained from Dr. H. Jakob (Institut Pasteur, Paris, France) were cultured in 100-mm dishes coated with gelatin in 10 mL Dulbecco's modified essential medium (with 4.5 g/L of D-glucose), containing 15% (v/v) fetal bovine serum, 50 units/mL of penicillin, 50 μg/mL of streptomycin, and 4mM L-glutamine.

Labeling of cells. — Exponentially growing cells were labeled for 24 h with D-[2-^{3}H]mannose (10 μCi/mL) or with D-[6-^{3}H]galactose (2.5 μCi/mL) in 10 mL of Dulbecco's modified essential medium (with 1 g/L of glucose), supplemented with 15% dialyzed fetal bovine serum, 50 units/mL of penicillin, 50 μg/mL of streptomycin, 4mM L-glutamine, and 2mM L-fucose. The inhibitors were added 2 h, and L-fucose was added 30 min before the labeled precursor. After labeling, the medium was removed, 3 mL of methanol were added to the dishes, the cells were scraped, transferred with 3 mL of methanol into tubes, and chloroform was added to yield 2:1 (v/v) chloroform–methanol. The pellet was extracted successively with 2 mL of 2:1 (v/v) chloroform–methanol, 0.5 mL of methanol, 3 mL of water (5 times), 0.5 mL of methanol, and 3 mL of chloroform–methanol–water (10:10:3, by vol., 3 times).

Preparation of glycopeptides and fractionation of oligosaccharides. — The lipid-free residue was exhaustively digested with pronase and the resulting glycopeptides were chromatographed on a column of Bio-Gel P-6, before and after endo H treatment as described previously[24]. The endo H-sensitive oligosaccharides were pooled, concentrated, and chromatographed with [^{14}C]-labeled $Glc_{1-3}Man_9GlcNAc$ standards on a column (25 × 0.46 cm) of 5 μm Aminospherisorb (Phase Separations, packed by Chromatography Sciences Co., Ville Mont Royal, Quebec, Canada) with a Varian model 5000 liquid chromatograph as described previously[14]. Exhaustive treatment of the oligosaccharides with jack bean α-D-mannosidase was performed as described previously[14]. The products were then subjected to l.c. as already described. [^{14}C]-Labeled $Glc_{1-3}Man_9GlcNAc$ standards were also treated with jack bean α-D-mannosidase to identify $Glc_{1-3}Man_4GlcNAc$ oligosaccharides.

Acid hydrolysis. — Samples were hydrolyzed with 0.5M H_2SO_4 for 7 h at 100°. The hydrolyzates were desalted through columns of AG50W (X8, H^+ form, 200–400 mesh) and AG1 (X8, formate form, 200–400 mesh) resins and were subjected to paper chromatography in ethyl acetate–2-propanol–water (65:23:12, by vol.) as described previously[14].

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