Asparagine-linked oligosaccharides of BHK cells treated with inhibitors of oligosaccharide processing

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Baby-hamster kidney (BHK) cells were labelled with [2-³H]mannose for 1–2 days in media containing 1-deoxynojirimycin, *N*-methyl-1-deoxynojirimycin or 1-deoxymannojirimycin. Glycopeptides obtained by Pronase digestion of disrupted cells were analysed by lectin affinity chromatography, by Bio-Gel P4 gel filtration and by paper chromatography of oligosaccharides released by endo- β -*N*-acetylglucosaminidase H. Biosynthesis of complex-type oligosaccharides was diminished but not abolished, the greatest effect being obtained by continuous culture of cells with 1-deoxymannojirimycin. Under these conditions cells contained only 20–30 % of the concentration of complex-type chains found in control cells and correspondingly increased amounts of oligomannose-type chains. Similar concentrations of asparagine-linked Man₆-GlcNAc₂ and Man₅GlcNAc₂ were present in 1-deoxymannojirimycin-treated cells and control cells, indicating that the inhibition of complex-type chain formation was not related simply to an inability of inhibitor-treated cells to carry out extensive mannosidase-catalysed processing. *N*-Methyl-1-deoxynojirimycin induced accumulation of oligomannose-type chains containing three glucose residues, and cells treated with 1-deoxynojirimycin contained oligosaccharides with one to three glucose residues. Cells cultured in the presence of the inhibitors retained sensitivity towards the galactose-binding lectins ricin and modeccin.

INTRODUCTION

Inhibitors of specific steps in assembly of asparagine-linked oligosaccharides (*N*-glycans) are useful reagents to study the metabolic pathways of protein glycosylation and the biological roles of the sugar moieties of glycoproteins [1,2]. Previously we have examined the effects of swainsonine, an inhibitor of mannosidase II, on processing of *N*-glycans in BHK cells [3]. Incubation of the cells with swainsonine was found to induce a complete switch from assembly of complex-type *N*-glycans normally present to hybrid-type chains containing five mannose residues and NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 2/4 sequences attached to the Man α 1 $\rightarrow$ 3-linked residue of the core. Similar structures were identified in ricin-resistant mutants of BHK cells deficient in mannosidase II [4]. These results showed that under normal growth conditions BHK cells do not utilize alternative pathways independent of mannosidase II [5–7] for conversion of oligomannose-type chains into complex-type carbohydrate units.

In the present paper we report on the effects of other inhibitors of processing reactions on glycoprotein synthesis in BHK cells. 1-Deoxynojirimycin (dNM) and *N*-methyl-1-deoxynojirimycin (M-dNM) are analogues of D-glucose that inhibit competitively the processing enzymes glucosidases I and II [8–10]. 1-Deoxymannojirimycin (dMM), an analogue of D-mannose, is an inhibitor of some processing mannosidases [11–13]. Other studies with these inhibitors have used as probes of glycosylation, virally infected cells [11,13–15] and glycoproteins secreted by inhibitor-treated cells [8,16–18]. The

systems are selective in the sense that assembly of mature infective virus or secretion of glycoproteins may require glycoproteins bearing a normal complement of asparagine-linked oligosaccharides and may underestimate the effect of processing inhibitors on bulk protein glycosylation. Therefore we have examined the effects of dNM, M-dNM and dMM on protein glycosylation in BHK cells under conditions used previously [3] for swainsonine. We show that complex-type chains are present even when the cells are cultured for prolonged periods in medium containing the inhibitors.

EXPERIMENTAL

Materials

Materials were obtained as follows: concanavalin A–Sephacrose and lentil lectin–Sephacrose from Pharmacia; Bio-Gel P2 (100–200 mesh) and Bio-Gel P4 (< 400 mesh) from Bio-Rad Laboratories; Pronase CB and jack-bean α -mannosidase from Calbiochem–Behring; endo- β -*N*-acetylglucosaminidase H from Miles Laboratories; methyl α -glucoside and methyl α -mannoside from Sigma Chemical Co.; [2-³H]mannose (13.4 Ci/mmol) and [³⁵S]methionine (1.34 Ci/mmol) from Amersham International. dNM was a gift from Bayer A.G., Wuppertal-Elberfeld, Germany; M-dNM was prepared by *N*-methylation of dNM [2]; dMM was synthesized as described previously [11]; castanospermine from seeds of *Castanospermum australe* was provided by Dr B. Winchester, London, U.K. Each inhibitor was stored at 2 °C as 2 mg/ml solutions in water and sterilized by passage through 0.22 μ m-pore-size Millipore filters.

Abbreviations used: dNM, 1-deoxynojirimycin; M-dNM, *N*-methyl-1-deoxynojirimycin; dMM, 1-deoxymannojirimycin; BHK cells, baby-hamster kidney cells.

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Cell cultures

BHK cells were grown routinely in monolayer culture at 37 °C in Eagle's medium (Gibco, Paisley, Scotland, U.K.) containing 22 mM-glucose and supplemented with 10 % (v/v) foetal-calf serum, penicillin and streptomycin. Cell monolayers were trypsinized and re-seeded at 1:4 dilutions for further growth. For treatment of cell cultures with inhibitors, cells were grown in 100 mm-diameter plastic tissue-culture dishes containing 10 ml of a modified Eagle's medium containing 5 mM-glucose and supplemented as described above. The cells were grown to touching confluency before addition of inhibitor to the required concentration. After a further period of incubation to allow the inhibitors to take effect, [³H]mannose was added to give 2–10 µCi/ml and the cells were incubated for 24–48 h at 37 °C. The inhibitors were present during the labelling period.

Preparation of glycopeptides

Cells in monolayer cultures were washed with phosphate-buffered saline (0.15 M-NaCl/50 mM-sodium phosphate buffer, pH 7.2), harvested by scraping and digested with Pronase [19]. Washed cell pellets were suspended in 10 mM-CaCl₂/0.02 % NaN₃/50 mM-Tris/HCl buffer, pH 7.5 (0.5 ml), and heated at 90 °C for 5 min. Pronase solution (0.4 % in the above buffer) was added in 50 µl portions each day for 2–3 days during incubation at 37 °C. The digests were again heated to inactivate Pronase and centrifuged at 10000 g for 10 min. At least 85 % of total radioactivity incorporated into acid-precipitable cellular proteins was recovered in the supernatants after Pronase digestion.

Column chromatography

Glycopeptide solutions (0.5 ml or less) were applied at room temperature to columns (3 ml bed volume) of concanavalin A-Sepharose mounted in a multiple-capacity frame. After application of glycopeptides, the columns were sealed and kept for 1 h to allow maximal binding of glycopeptides. Then 1 ml fractions were collected manually by elution [19] first with 1 mM-CaCl₂/1 mM-MgCl₂/1 mM-MnCl₂/0.02 % NaN₃/10 mM-Tris/HCl buffer, pH 7.5 (10 ml), followed by 10 mM-methyl α-glucoside (10 ml) and 500 mM-methyl α-mannoside (10 ml) in the same buffer. Samples of column fractions were taken for counting of radioactivity. Up to six glycopeptide mixtures could conveniently be analysed simultaneously by this procedure. Appropriate column fractions were pooled, concentrated by rotary evaporation and desalted by passage through a column (2 cm × 27 cm) of Bio-Gel P2. Fractions (2 ml) eluted with water were collected, and peak radioactive fractions were dried and reconstituted in water (0.5 ml). Affinity chromatography of glycopeptides (0.5 ml or less) on lentil lectin-Sepharose column (3 ml bed volume) was performed [19] as described for concanavalin A-Sepharose chromatography except the elution employed first buffer (10 ml) followed by 200 mM-methyl α-glucoside in buffer (10 ml). Glycopeptides and oligosaccharides were also separated by chromatography at 2 °C on a column (1.2 cm × 90 cm) of Bio-Gel P4. Columns were run in 0.1 M-NH₄HCO₃, and 0.05–0.2 ml portions of glycopeptide or oligosaccharide mixtures were applied. Column fractions (1 ml) were collected at 5 ml/h, and appropriate peak radioactive fractions were pooled, freeze-dried and reconstituted in water (0.5 ml).

Paper chromatography

[³H]Mannose-labelled samples containing 1000–5000 c.p.m. were applied in 20 µl or less to Whatman no. 1 paper sheets and run in ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.) for various periods. Standard [³H]mannose-labelled compounds of known structure were included in each separation. Each sample track after development was cut into 1 cm segments, the segments were eluted with 1 ml of water and samples were counted for radioactivity.

Glycosidase treatments of glycopeptides and oligosaccharides

Salt-free glycopeptide fractions were freeze-dried, redissolved in 100 mM-sodium citrate/phosphate buffer, pH 5.5 (90 µl), and digested with 5–10 units (5–10 µl) of endo-β-N-acetylglucosaminidase H at 37 °C for 2 days. Other glycopeptide or oligosaccharide fractions were treated in 90 µl of 0.3 mM-ZnSO₄/0.1 M-sodium acetate buffer, pH 5, with 10 µl of jack-bean α-mannosidase at 37 °C for 24–48 h.

Oligosaccharide standards

[³H]Mannose-labelled oligosaccharides Man₆GlcNAc to Man₉GlcNAc were obtained from mouse parietal endoderm PYS cells [20] or ricin-resistant BHK-cell mutants Ric^R14 and Ric^R21 [19,21]. Briefly, high-mannose glycopeptide fractions, prepared from these cells grown for 2–3 days in medium containing [2-³H]-mannose, were treated with endo-β-N-acetylglucosaminidase H and oligosaccharides were isolated by preparative paper chromatography. A mixture of [³H]-mannose-labelled oligosaccharides was also kindly supplied by Dr R. Kornfeld, Washington University, St. Louis, MO, U.S.A.

Lectin toxicity

Cells grown in control medium or in media containing 2 mM-dNM, -dMM or -castanospermine were trypsinized, seeded on 35 mm-diameter dishes and grown to confluency in media supplemented as above. The growth medium was then removed and replaced with either ricin or modeccin (0–100 µg/ml) in serum-free medium (1 ml) with or without glycosylation inhibitors. After incubation at 37 °C for 3 h the cells were labelled for 1 h at 37 °C with [³⁵S]methionine (0.1 ml, 20 µCi). Incorporation of radioactivity into acid-precipitable material was measured as described previously [3].

RESULTS

Concanavalin A-Sepharose chromatography of [³H]mannose-labelled glycopeptides

Glycopeptides obtained from BHK cells by Pronase digestion after metabolic labelling with [³H]mannose were separated into three fractions by chromatography on concanavalin A-Sepharose (Fig. 1). Fraction A eluted with buffer contains tri- and tetra-antennary complex-type glycopeptides, fraction B eluted with 10 mM-methyl α-glucoside contains bi-antennary complex-type glycopeptides, and fraction C eluted with 500 mM-methyl α-mannoside contains oligomannose-type and hybrid-type glycopeptides [3,19,21,22]. All three fractions were obtained from BHK cells treated with 1 mM-dNM (Fig. 1a), 1 mM-M-dNM (Fig. 1b) or 1 mM-dMM (Fig.

Fig. 1. Separation of BHK-cell glycopeptides on concanavalin A-Sepharose

$[^3\text{H}]\text{Mannose}$ -labelled glycopeptides were prepared from control cells or cells treated with various inhibitors (1 mM) at 37°C for 1 h before addition of label and further incubation for 24 h. The glycopeptides were eluted from concanavalin A-Sepharose with buffer, 10 mM-methyl α -glucoside in buffer and 500 mM-methyl α -mannoside in buffer as indicated by the arrows. Fractions A, B and C were pooled. Cells were treated with: (a) dNM; (b) M-dNM; (c) dMM; (d) no inhibitor. Approx. 2×10^5 c.p.m. of each sample was used for chromatography.

Fig. 2. Effect of various concentrations of inhibitors on glycopeptide composition of BHK cells

The relative amounts of radioactivity recovered in fractions A (∇), B (Δ) and C ($\circ$) from concanavalin A-Sepharose (see Fig. 1) are shown. Inhibitors were: (a) dNM; (b) M-dNM; (c) dMM. The values obtained for untreated cells labelled for 24 h with $[^3\text{H}]\text{mannose}$ are shown by black symbols in panel (a). Recovery of radioactivity from the columns was at least 70% in all cases.

1c), although in different relative amounts compared with control cells (Fig. 1d). Glycopeptides from dMM-treated cells were recovered largely (88%) in fraction C, and fractions A and B were in decreased amounts relative to control cell glycopeptides. In Pronase digests of cells treated with either 1 mM-dNM or 1 mM-M-dNM

the proportion particularly of tri- and tetra-antennary glycopeptides was decreased compared with control cell glycopeptides. Bi-antennary complex-type glycopeptides were less affected. The changes in glycopeptide composition were dependent on inhibitor concentration (Fig. 2).

Fig. 3. Effect of medium glucose concentration on dNM-induced changes in glycopeptide composition of BHK cells

Cells were grown to confluency in medium containing 22 mM-glucose, then kept in media containing the stated glucose concentrations and 2 mM-dNM for 6 h at 37 °C before labelling with [³H]mannose for 24 h at 37 °C. Glycopeptide fractions A–C were obtained as described in Fig. 1.

Table 1. Lentil lectin–Sephacrose chromatography of concanavalin A–Sephacrose fractions

Glycopeptide fractions from cells treated for 1 h at 37 °C with 2 mM inhibitor before labelling with [³H]mannose and isolated as shown in Fig. 1 were applied to 3 ml columns of lentil lectin–Sephacrose. Fractions (1 ml) were eluted with buffer (10 ml) followed by 200 mM-methyl α -glucoside in buffer (10 ml) and counted for radioactivity. Approx. 1000 c.p.m. of each glycopeptide fraction was applied to lentil lectin–Sephacrose.

Inhibitor	Glycopeptide	Percentage bound
dNM	A	16.2
	B	63.1
M-dNM	A	16.9
	B	62.9
dMM	A	3.8
	B	60.1
None	A	13.9
	B	74.6

Effect of glucose concentration in the medium

The standard medium used for the above experiments contains 5 mM-glucose. Since dNM and M-dNM are simple structural analogues of D-glucose, the ring oxygen atom being replaced by a nitrogen function [2], one possible explanation for the apparently incomplete inhibition of processing of asparagine-linked oligosaccharides in cells treated with these substances may be a competitive inhibition of uptake by medium glucose. However, as Fig. 3 shows complex-type glycopeptides were still obtained from cells treated with 2 mM-dNM in media containing amounts of glucose as low as 0.2 mM.

Fig. 4. Bio-Gel P4 chromatography of endo- β -N-acetylglucosaminidase H-treated glycopeptides

BHK cells were treated for 1 h at 37 °C with 1 mM-dNM (a), 1 mM-M-dNM (b) or 1 mM-dMM (c) before labelling with [³H]mannose for 40 h. Glycopeptide fractions C (see Fig. 1) were treated with endo- β -N-acetylglucosaminidase H, applied separately to a calibrated Bio-Gel P4 column and 1 ml fractions were eluted with 0.1 M-NH₄HCO₃. Samples were taken for counting of radioactivity. Approx. 80000 c.p.m. of each sample was used for chromatography. The arrows show the elution of Man₉GlcNAc (9), Man₅GlcNAc (5) and mannose (V_t) respectively. Column fractions pooled for further analysis are indicated by bars.

Analysis of complex-type glycopeptides

Evidence supporting the presence of complex-type glycopeptides in concanavalin A–Sephacrose fractions A and B (Fig. 1) from inhibitor-treated cells was obtained. The glycopeptides were eluted from a calibrated Bio-Gel P4 column coincidentally with standard complex-type

Fig. 5. Paper chromatography of [^3H]mannose-labelled oligosaccharides

Bio-Gel P4 fractions from Fig. 4 were subjected to paper chromatography for various times. The paper was cut into 1 cm strips and each strip was eluted with 1 ml of water. Samples were counted for radioactivity. (a)–(c) Bio-Gel P4 fractions 1, 2 and 3 respectively from cells treated with dNM (Fig. 4a); (d)–(e) Bio-Gel P4 fractions 1 and 2 respectively from cells treated with M-dNM (Fig. 4b); (f)–(g) Bio-Gel P4 fractions 1 and 2 from cells treated with dMM (Fig. 4c). The migration of standards $\text{Man}_9\text{GlcNAc}$ to $\text{Man}_5\text{GlcNAc}$ is indicated by arrows (9–5). The times used for chromatography of samples was 7 days (a and b), 3 days (c) or 4 days (d–g). In panels (a) and (b) fractions I–IV were pooled for further analysis.

glycopeptides from control BHK cells [19] and were resistant to endo- β -*N*-acetylglucosaminidase H treatment (results not shown). On lentil lectin–Sephacrose chromatography similar proportions of the [^3H]mannose radioactivity present in glycopeptide fractions A and B obtained from control, dNM-treated or M-dNM-treated cells were bound (Table 1), indicating similar extents of core fucosylation [19]. Concanavalin A–Sephacrose fraction B from dMM-treated cells also behaved similarly to fraction B obtained from control cells on lentil lectin–Sephacrose chromatography, but fraction A appeared to contain a smaller amount of core-fucosylated glycopeptides (Table 1).

Analysis of oligomannose-type glycopeptides

All of the [^3H]mannose-labelled glycopeptides present in concanavalin A–Sephacrose fractions C (Fig. 1) from control or inhibitor-treated cells were sensitive to endo- β -*N*-acetylglucosaminidase H treatment. Chromatography on a calibrated Bio-Gel P4 column showed that the endo- β -*N*-acetylglucosaminidase H-released products were eluted as components of lower molecular size compared with the untreated glycopeptides. The results of Bio-Gel P4 chromatography of the endo- β -*N*-acetylglucosaminidase H-released oligosaccharides are shown in Fig. 4.

dMM-treated cells. The predominant [^3H]mannose-labelled oligosaccharides obtained from dMM-treated cells were eluted from Bio-Gel P4 in a broad peak with a size similar to a $\text{Man}_9\text{GlcNAc}$ standard, but smaller oligosaccharides were also present in significant amounts (Fig. 4c). Further identification of the oligosaccharides was obtained by paper chromatography. Bio-Gel P4 fraction 1 contained components co-migrating with standards of $\text{Man}_9\text{GlcNAc}$ and $\text{Man}_8\text{GlcNAc}$ (Fig. 5f), and fraction 2 contained, in addition to $\text{Man}_9\text{GlcNAc}$, a major $\text{Man}_7\text{GlcNAc}$ peak and smaller amounts of $\text{Man}_6\text{GlcNAc}$ and $\text{Man}_5\text{GlcNAc}$ (Fig. 5g). Oligosaccharide fractions 1 and 2 were digested completely by jack-bean α -mannosidase to free mannose and a minor component that was eluted in column fractions 88–93, in the position of $\text{Man}\beta 1\rightarrow 4\text{GlcNAc}$ (Fig. 6b). The recoveries of [^3H]mannose radioactivity in these two peaks were in the ratios 7.9:1 for fraction 1 (Fig. 6b) and 6.6:1 for fraction 2 (results not shown), consistent with the oligosaccharide compositions determined by paper chromatography and assuming equal labelling of all mannose residues. A quantitative determination of the relative concentrations of the various oligosaccharides was obtained by integration of the areas under each peak separated by paper chromatography and making appropriate corrections for the number of mannose residues

Fig. 6. Bio-Gel P4 chromatography of α -mannosidase-treated oligosaccharides

Oligosaccharide fractions obtained as described in Fig. 4 and containing approx. 2500 c.p.m. of [3 H]mannose were treated with jack-bean α -mannosidase before re-chromatography. Column fractions (1 ml) were collected, mixed with scintillation fluid and counted for radioactivity. (a) Fraction 1 (●—●) or 2 (— — —) from M-dNM-treated cells (see Fig. 4b); (b) fraction 1 (●—●) from dNM-treated cells (see Fig. 4c); similar results were obtained with fraction 2.

present. Similar treatment was performed for the complex-type glycopeptides eluted from concanavalin A-Sepharose in fractions A and B (Fig. 1c) and assumed to contain a trimannosyl core sequence. Table 2 (Expt. 1) presents the overall composition of asparagine-linked oligosaccharides obtained from this analysis and similar analysis of control BHK-cell glycopeptides for comparison.

M-dNM-treated cells. The endo- β -N-acetylglucosaminidase H-sensitive oligosaccharides obtained from cells treated with M-dNM contained components larger than $\text{Man}_6\text{GlcNAc}$, as shown by their earlier elution from Bio-Gel P4 (Fig. 4b). Bio-Gel P4 fraction 2 gave on paper chromatography oligosaccharides migrating with $\text{Man}_6\text{GlcNAc}$ to $\text{Man}_5\text{GlcNAc}$ standards and additional material migrating more slowly than $\text{Man}_6\text{GlcNAc}$ (Fig. 5e). Fraction 1 consisted mainly of slow-moving oligosaccharides (Fig. 5d). After paper chromatography of fraction 1 for 8 days three distinct components were detected (results not shown). The most likely com-

Table 2. Asparagine-linked oligosaccharide composition of control and dMM-treated BHK cells

The percentage recovery of [3 H]mannose radioactivity in each fraction isolated by sequential concanavalin A-Sepharose, Bio-Gel P4 and paper chromatography (see Figs. 1, 4 and 5) was converted into percentage by weight of each component by correcting for the known number (n) of mannose residues (three for complex-type chains) and assuming equal labelling of all mannose residues, according to the formula $(\text{peak area}/n) \div \sum(\text{peak areas}/n) \times 100$. Expt. 1, 1 h pretreatment with 1 mM-dMM before metabolic labelling; Expt. 2, cells cultured continuously in 2 mM-dMM before labelling.

Concanavalin A-Sepharose fraction	Oligosaccharide composition (% of total)		
	Control	+ dMM	
		Expt. 1	Expt. 2
A	62.6	30.1	20.0
B	18.0	10.0	6.9
C			
$\text{Man}_6\text{GlcNAc}$	2.3	10.8	30.0
$\text{Man}_8\text{GlcNAc}$	2.6	16.1	17.5
$\text{Man}_7\text{GlcNAc}$	3.1	15.7	10.0
$\text{Man}_6\text{GlcNAc}$	7.7	12.4	8.1
$\text{Man}_5\text{GlcNAc}$	3.5	4.8	6.8

positions for these components are $\text{Glc}_3\text{Man}_6\text{GlcNAc}$, $\text{Glc}_3\text{Man}_8\text{GlcNAc}$ and $\text{Glc}_3\text{Man}_7\text{GlcNAc}$ for the following reasons. Treatment of Bio-Gel fraction 1 (Fig. 4b) with jack-bean α -mannosidase released about 50% of the label as free mannose and produced modified oligosaccharides that were eluted from Bio-Gel P4 in a major peak and a minor peak representing 54% and 6% of total radioactivity respectively (Fig. 6a). The presence of glucose protects from α -mannosidase action mannose residues substituted on the $\alpha 1 \rightarrow 3$ -linked mannose unit of the core sequence. The shift in size of the major resistant fragment of fraction 1 oligosaccharides and the proportion of label released as free mannose suggests that removal of three or four mannose units had occurred. The resistant oligosaccharides retained affinity for concanavalin A-Sepharose, as shown by re-chromatography, when all of the radioactivity associated with these oligosaccharides was bound and eluted with 500 mM-methyl α -mannoside (results not shown). Evidently the $\alpha 1 \rightarrow 6$ -linked mannose residue of the core sequence was not removed by jack-bean α -mannosidase, and hence the end products retained two reactive terminal α -mannose residues required for concanavalin A binding. Previously we have reported the resistance of this residue to α -mannosidase in hybrid-type oligosaccharides containing substituents on the $\alpha 1 \rightarrow 3$ -linked mannose residue of the core sequence [23]. It seems likely from the results that the major mannosidase-resistant oligosaccharide (Fig. 6a) was $\text{Glc}_3\text{Man}_5\text{GlcNAc}$ and that the minor resistant oligosaccharide component contained one or two fewer glucose residues. Fraction 2 (Fig. 4b) produced after jack-bean α -mannosidase treatment mainly free mannose and a small peak of $\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}$ (Fig. 6a), confirming that it consists largely of non-glucosylated species.

dNM-treated cells. Bio-Gel P4 fraction 3 obtained from dNM-treated cells (Fig. 4a) consisted mainly of $\text{Man}_6\text{GlcNAc}$, $\text{Man}_7\text{GlcNAc}$ and $\text{Man}_8\text{GlcNAc}$ with smaller amounts of $\text{Man}_9\text{GlcNAc}$ and $\text{Man}_5\text{GlcNAc}$ (Fig. 5c). Fraction 2 contained minor proportions of $\text{Man}_9\text{GlcNAc}$ and $\text{Man}_8\text{GlcNAc}$, a major component IV migrating between these oligosaccharides and another major component III migrating more slowly than $\text{Man}_9\text{GlcNAc}$ (Fig. 5b). Fraction 1 mainly contained two slowly migrating oligosaccharides I and II (Fig. 5a). From their migration relative to $\text{Man}_9\text{GlcNAc}$ on paper chromatography, oligosaccharides I–III probably contain one to three additional hexose units. Evidence that these components and oligosaccharide IV represent glucosylated $\text{Man}_n\text{GlcNAc}$ species containing variable amounts of glucose and mannose was obtained by digestion with jack-bean α -mannosidase and analysis of products on Bio-Gel P4. Oligosaccharide I is probably $\text{Glc}_3\text{Man}_9\text{GlcNAc}$, since mannosidase released 42 % of label as free mannose and produced a resistant fragment that was eluted from Bio-Gel P4 in the position (column fractions 65–70) described above for a $\text{Glc}_3\text{Man}_5\text{GlcNAc}$ species (see Fig. 6a). Similar treatment of oligosaccharides II, III and IV with jack-bean α -mannosidase released free mannose and resistant oligosaccharide fragments that were eluted from Bio-Gel P4 in column fractions 65–70 and in a fraction (see Fig. 6a) of lower size (column fraction 72–77). The relative proportion of radioactivity recovered in the small resistant fragment was greatest in oligosaccharide IV, where it was the major resistant component. Thus the available evidence suggests that the major component of oligosaccharide IV is probably $\text{Glc}_2\text{Man}_7\text{GlcNAc}$, III contains a mixture of $\text{Glc}_2\text{Man}_8\text{GlcNAc}$ and $\text{Glc}_3\text{Man}_8\text{GlcNAc}$, and II contains probably a mixture of $\text{Glc}_2\text{Man}_6\text{GlcNAc}$ and $\text{Glc}_3\text{Man}_6\text{GlcNAc}$.

Effects of long-term treatments with inhibitors on asparagine-linked oligosaccharides of BHK cells

In an attempt to induce more extreme effects of the inhibitors on oligosaccharide processing, experiments were performed in which cells were cultured continuously in media containing either 2 mM-dNM or 2 mM-dMM. The cells maintained full viability during such treatments, and no effects were detected on growth rate or plating efficiency after four successive sub-cultures (16–20 cell divisions) over a period of approx. 3 weeks. No morphological changes were observed in the treated cells (results not shown). Cells cultured continuously with inhibitors were labelled metabolically with [^3H]mannose for 48 h in the presence of inhibitors, and glycopeptides generated with Pronase were analysed by concanavalin A–Sephadex chromatography. The recoveries of [^3H]mannose radioactivity in fractions A, B and C respectively were as follows: dNM-treated cells, 27.8 %, 15.7 % and 56.4 %; dMM-treated cells, 9.6 %, 3.2 % and 87.2 %. These results are very similar to those obtained with short (1–6 h) periods of exposure of cells to the inhibitors before [^3H]mannose labelling (Fig. 2). In particular, formation of complex-type chains was not totally prevented. However, the effect of prolonged treatment with dMM was somewhat more pronounced than was obtained after short-term exposure, as shown by analysis of the oligosaccharides released from concanavalin A–Sephadex fraction C with endo- β -N-acetylglucosaminidase H (Table 2, Expt. 2). There was a

significant decrease in relative concentrations of complex-type glycopeptide fractions A and B, and $\text{Man}_9\text{GlcNAc}$ and $\text{Man}_8\text{GlcNAc}$ accounted for over 80 % of the oligomannose-type structures.

Sensitivity to toxic lectins of cells treated with processing inhibitors

BHK cells cultured continuously in media containing 2 mM concentrations of dNM, dMM and castanospermine, an inhibitor of processing glucosidases [1,2], were incubated with the plant toxins ricin and modeccin. These toxins bind to galactose residues of asparagine-linked oligosaccharides present in cell-surface glycoproteins, and this binding is a necessary first step in mediation of lectin cytotoxicity. As shown in Fig. 7, the resistance of BHK cells to ricin, as measured by incorporation of [^{35}S]methionine into total cellular proteins, was only marginally increased by pretreatment of the cells with the processing inhibitors. The protective effect against modeccin cytotoxicity was more significant. In both cases dMM produced the largest protective effect, consistent with the most profound inhibitory effect of this inhibitor on the assembly of complex-type asparagine-linked oligosaccharides containing galactose. Castanospermine gave the next most protection, whereas dNM gave little or no protection.

DISCUSSION

The present results show that treatment of BHK cells under various conditions with dNM or M-dNM influenced the composition of asparagine-linked oligosaccharides in bulk cellular glycoproteins but did not prevent formation of complex-type oligosaccharides. Both inhibitors induced accumulation of glucosylated oligomannose units as stable entities in glycoproteins without affecting the overall extent of bulk glycosylation, since the extent of incorporation of [^3H]mannose into glycoproteins was similar over 1–2 days of labelling in the inhibitor-treated or control cells. Hence it can be excluded that in the inhibitor-treated cells we were measuring the glycosylation of a sub-set of cellular glycoproteins that under normal conditions also carried glucosylated carbohydrate chains.

On the basis of the preliminary structural information obtained with endo- β -N-acetylglucosaminidase H-released material, M-dNM appears to induce accumulation of three oligosaccharides migrating more slowly than $\text{Man}_9\text{GlcNAc}$ in paper chromatography. Jack-bean α -mannosidase digestion of these oligosaccharides gave a major product with characteristics expected of the mannosidase-resistant fragment of $\text{Glc}_3\text{Man}_6\text{GlcNAc}$, namely $\text{Glc}_3\text{Man}_5\text{GlcNAc}$, and only minor amounts of smaller oligosaccharides containing fewer glucose units. The results suggest that the three slowly migrating oligosaccharides are $\text{Glc}_3\text{Man}_9\text{GlcNAc}$, $\text{Glc}_3\text{Man}_8\text{GlcNAc}$ and $\text{Glc}_3\text{Man}_7\text{GlcNAc}$, similar to findings reported in other studies [14,18]. The oligosaccharides released by endo- β -N-acetylglucosaminidase H from dNM-treated cells appear to include species containing fewer than three glucose residues, since the products of jack-bean α -mannosidase digestion contained, in addition to $\text{Glc}_3\text{Man}_5\text{GlcNAc}$, smaller resistant oligosaccharide fragments of probable structure $\text{Glc}_2\text{Man}_5\text{GlcNAc}$ and $\text{Glc}_1\text{Man}_5\text{GlcNAc}$. The fact that extensive processing continues in inhibitor-treated cells, even

Fig. 7. Effect of glycosylation inhibitors on the sensitivity of BHK cells to modeccin (a) and ricin (b)

Cells were grown to confluency in control medium or medium containing 2 mM glycosylation inhibitor. After reaching confluency the cells were treated with either modeccin or ricin at the concentrations indicated. Treatment at 37 °C was for 1 h before addition of [³⁵S]methionine and incorporation into acid-insoluble proteins was measured after a further 1 h incubation. Cells treated with: ○, castanospermine; ●, dNM; ▲, dMM; △, no inhibitor.

producing Man₅GlcNAc in significant amounts, indicates either that a low residual activity of glucosidases I and II persists or that alternative metabolic pathways operate which by-pass these enzyme activities. Assays *in vitro* have shown that glucosidase I is strongly inhibited by M-dNM (50 % inhibition at 0.07 μM concentration) and less inhibited by dNM (1–3 μM concentrations) whereas glucosidase II is inhibited only at concentrations above 20 μM [8–10]. Although we would expect these concentrations to be reached by treatment of cells with 1–2 mM inhibitor, this may not be the case, especially for dNM. Evidence for transfer of non-glycosylated oligosaccharides to polypeptide has recently been reported in F9 teratocarcinoma cells exposed to M-dNM [24].

Our data obtained with dMM also show that extensive processing occurred in cells treated with this inhibitor. Several processing mannosidases specific for removal of α1→2-linked mannose residues of the Glc₃Man₉-GlcNAc₂ precursor have been described in endoplasmic reticulum and Golgi membranes [2]. As shown by assays *in vitro*, dMM inhibits rat liver Golgi mannosidases IA and IB [12] and a calf liver microsomal Man₉ mannosidase [13] at sub-micromolar concentrations. If these dMM-sensitive enzymes were maximally inhibited in BHK cells treated with 1–2 mM concentrations of dMM used in our experiments, the observed processing could involve an endoplasmic-reticulum mannosidase that, as Bischoff & Kornfeld [12] have shown, is not inhibited by dMM. In rat liver the endoplasmic-reticulum mannosidase is thought to remove specifically only one α1→2-linked mannose residue from Man₉GlcNAc₂ [12,25,26], although the solubilized enzyme shows a broader substrate specificity [26], which in BHK cells may be expressed sufficiently to produce the extensive processing observed. However, the possible presence of

additional dMM-insensitive mannosidases active on Man₈GlcNAc and smaller oligomers cannot be excluded.

Several additional points arise from the analysis shown in Table 2. First, the absolute concentrations of Man₅GlcNAc and Man₆GlcNAc are similar in control or dMM-treated cells, and hence the decreased amount of complex-type oligosaccharides in dMM-treated cells cannot be related simply to lack of a suitable substrate (Man₅GlcNAc₂) for enzymes involved in conversion of oligomannose-type units into complex-type chains. This assumes that the fully processed oligomannose units appear on nascent glycoproteins at appropriate intracellular sites containing the converting glycosyltransferases and that these latter enzymes are correctly positioned in dMM-treated cells, neither of which assumption may be true. However, it is clear that the major switch in dMM-treated cells involves the larger-sized oligomannose units. In dMM-treated cells the decrease in concentration of complex-type chains is balanced approximately by an increased concentration of Man₉GlcNAc, Man₈GlcNAc and Man₇GlcNAc species (Table 2). Presumably, since the greatest effect of dMM was observed on the concentration of the Man₉-GlcNAc species (a 5–15-fold increase, Table 2), at least part of the initial processing of protein-linked Man₉-GlcNAc₂ involves a dMM-sensitive endoplasmic-reticulum mannosidase and not the dMM-insensitive enzyme described by Bischoff & Kornfeld [12,26]. Similar evidence for distinct processing pathways in rat liver were recently obtained by Bischoff *et al.* [25]. It is possible that processing of oligomannose units destined for conversion into complex-type chains may be different, involving separate enzymes, to the processing leading to stable oligomannose-type units of mature glycoproteins. If this speculation is correct, the data collected in Table 2

imply that in BHK cells approx. 30% of complex-type oligosaccharides are assembled by a dMM-insensitive pathway, 50% are assembled by a pathway totally involving dMM-sensitive enzymes and 20% are assembled by a pathway involving the dMM-insensitive enzyme converting $\text{Man}_5\text{GlcNAc}_2$ into $\text{Man}_8\text{GlcNAc}_2$ [12,26] and dMM-sensitive processing mannosidases involved in further processing of the $\text{Man}_8\text{GlcNAc}_2$ intermediate.

The results described here obtained with inhibitors of processing glucosidases I and II (dNM and M-dNM) or mannosidases (dMM) are in striking contrast with an earlier study using swainsonine [3]. Swainsonine is an inhibitor of mannosidase II, which is responsible for hydrolysis of two mannose residues linked respectively $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 6$ in the biosynthetic asparagine-linked intermediate $\text{GlcNAcMan}_5\text{GlcNAc}_2$, and is a key enzyme in conversion of oligomannose-type and hybrid-type oligosaccharides into complex-type chains. Treatment of BHK cells with swainsonine was found to prevent completely formation of complex-type chains in bulk cellular glycoproteins, which were replaced quantitatively by hybrid-type chains. Cells bearing these modified carbohydrates were markedly more resistant to ricin cytotoxicity.

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