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The effect of castanospermine on the oligosaccharide structures of glycoproteins from lymphoma cell lines

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The effect of castanospermine on the processing of *N*-linked oligosaccharides was examined in the parent mouse lymphoma cell line and in a mutant cell line that lacks glucosidase II. When the parent cell line was grown in the presence of castanospermine at 100 μg/ml, glucose-containing high-mannose oligosaccharides were obtained that were not found in the absence of inhibitor. These oligosaccharides bound tightly to concanavalin A–Sepharose and were eluted in the same position as oligosaccharides from the mutant cells grown in the absence or presence of the alkaloid. The castanospermine-induced oligosaccharides were characterized by gel filtration on Bio-Gel P-4, by h.p.l.c. analysis, by enzymic digestions and by methylation analysis of [^{3}H]mannose-labelled and [^{3}H]galactose-labelled oligosaccharides. The major oligosaccharide released by endoglucosaminidase H in either parent or mutant cells grown in castanospermine was a $Glc_3Man_7GlcNAc$, with smaller amounts of $Glc_3Man_8GlcNAc$ and $Glc_3Man_9GlcNAc$. On the other hand, in the absence of castanospermine the mutant produces mostly $Glc_2Man_7GlcNAc$. In addition to the above oligosaccharides, castanospermine stimulated the formation of an endoglucosaminidase H-resistant oligosaccharide in both cell lines. This oligosaccharide was characterized as a $Glc_2Man_5GlcNAc_2$ {i.e., Glc(1,2)Glc(1,3)Man(1,2)Man(1,2)Man(1,3)[Man(1,6)]Man-GlcNAc-GlcNAc}. Castanospermine was tested directly on glucosidase I and glucosidase II in lymphoma cell extracts by using [*Glc*-^{3}H]$Glc_3Man_9GlcNAc$ and [*Glc*-^{3}H]$Glc_2Man_9GlcNAc$ as substrates. Castanospermine was a potent inhibitor of both activities, but glucosidase I appeared to be more sensitive to inhibition.

The glycosylation of asparagine residues on plant and animal glycoproteins occurs by transfer of a $Glc_3Man_9GlcNAc_2$ from the lipid-linked oligosaccharide dolichyl pyrophosphate $Glc_3Man_9GlcNAc_2$ (Hemming, 1974; Lehle & Tanner, 1983), to the nascent protein (Hubbard & Ivatt, 1981). After this transfer the oligosaccharide undergoes a number of processing reactions that begin in the endoplasmic reticulum and continue in the Golgi apparatus (Grinna & Robbins, 1979, Turco & Robbins, 1979; Elting *et al.*, 1980).

The initial processing reactions involve the subsequent removal of the three glucose residues, catalysed by at least two different glucosidases (Chen & Lennarz, 1978; Spiro *et al.*, 1979; Ugalde *et al.*, 1978). Glucosidase I removes the outermost α1,2-linked glucose residue (Hettkamp *et al.*, 1984), whereas glucosidase II releases the next two α1,3-linked glucose units (Michael & Kornfeld, 1980; Burns & Touster, 1982). These trimming reactions give a $Man_9GlcNAc_2$-protein, which may be the immediate precursor to the high-mannose glycoproteins, or it may be processed further by the removal of four α1,2-linked mannoses by endoplasmic-reticulum- (Bischoff & Kornfeld, 1983) and Golgi-derived (Opheim & Touster, 1978; Tabas & Kornfeld, 1979; Forsee & Schutzbach, 1981; Tulsiani *et al.*, 1982) α-mannosidases. Then a GlcNAc transferase adds a GlcNAc residue to the α1,3-linked mannose residue that is attached to the β-linked mannose, and this

Abbreviation used: Endo H, endoglucosaminidase H.

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addition signals mannosidase II to remove the α1,3- and α1,6-linked mannoses from the 1,6-branch (Tabas & Kornfeld, 1978; Harpaz & Schachter, 1980). Thereafter, the other sugars of the complex chains can be added to form the final complex structure. Thus both the high-mannose and the complex types of glycoproteins are derived from the same intermediate, namely $Glc_3Man_9GlcNAc_2$ (Hubbard & Ivatt, 1981).

Previous studies from our laboratory have shown that the plant alkaloid castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizidine) is a potent inhibitor of fibroblast α- and β-glucosidase, as well as almond emulsin β-glucosidase (Saul *et al.*, 1983). In addition, castanospermine inhibited the processing of the oligosaccharide chain of the influenza-virus haemagglutinin when the virus was grown in MDCK (Madin–Darby canine kidney) cells in the presence of alkaloid. Since the major oligosaccharide that was present in the viral glycoproteins was a $Glc_3Man_7GlcNAc_2$, the data indicated that this alkaloid inhibited glucosidase I (Pan *et al.*, 1983; Elbein, 1984). In order to determine whether castanospermine would have the same effect in a non-viral system, it was tested as an inhibitor in a mouse lymphoma cell line that contained all the processing enzymes, and also in a mutant mouse lymphoma cell line that lacks glucosidase II (Reitman *et al.*, 1982). Although the results were generally similar to those with the influenza-virus haemagglutinin, i.e., the major endoglucosaminidase H-released oligosaccharide of parent and mutant lymphoma cell lines was a $Glc_3Man_7GlcNAc$, another unusual oligosaccharide was found in the presence of alkaloid. This structure was resistant to endoglucosaminidase H and was characterized as a $Glc_2Man_5GlcNAc_2$. In addition, we show that castanospermine inhibited both glucosidase I and glucosidase II from mouse lymphoma cells.

Materials and methods

Materials

[2-^{3}H]Mannose (sp. radioactivity 25Ci/mmol) and [1-^{3}H]galactose (12mCi/mmol) were purchased from Pathfinders Laboratories, St. Louis, MO, U.S.A. Endo-β-*N*-acetylglucosaminidase H was obtained from Health Research, Albany, NY, U.S.A. Pronase was from Calbiochem, jack-bean (*Canavalia ensiformis*) α-mannosidase and concanavalin A–Sepharose were from Sigma Chemical Co., and Bio-Gel P-4 was from Bio-Rad. Castanospermine was extracted in 0.3% yield from the seeds of *Castanospermum australe*, purified by repeated chromatography on Dowex 50 (NH_4^+ form) resin and crystallized from ethanol (Hohenschutz *et al.*, 1981).

Cell lines

The parent mouse lymphoma cell line (BW 5147) and the mutant cell line (PHA^{R}2.7) that is resistant to the cytotoxic effects of the leucoagglutinating agent from *Phaseolus vulgaris* (French bean), were kindly provided by Dr. Stuart Kornfeld (Washington University School of Medicine, St. Louis, MO, U.S.A.). The cells were grown in suspension culture, without agitation, in 10ml of Eagle's minimal essential medium, supplemented with 10% (v/v) calf serum as previously described (Reitman *et al.*, 1982).

Labelling of cells with radioactive precursors

Cells were grown as described above. Cells at a density of 2×10^5 cells/ml were usually labelled for 48h in 10ml of 2% basal minimal essential medium containing 100μCi of [2-^{3}H]mannose or [1-^{3}H]-galactose. When the effect of inhibitor was to be tested, various amounts of castanospermine were added several hours before the addition of the labelled compound to allow the inhibitor to take effect. The inhibitor was present during the labelling period. By the end of the labelling period, cells had reached a density of $(6–9) \times 10^5$ cells/ml and were 91–94% viable on the basis of Trypan Blue exclusion.

Preparation and isolation of glycopeptides

Cells were isolated by centrifugation, washed with phosphate-buffered saline (per litre of water: NaCl, 7.65g; Na_2HPO_4, 1.26g; NaH_2PO_4, 0.1g; KH_2PO_4, 0.21g), and digested exhaustively with Pronase. Glycopeptides were separated from salt and free sugars on columns of Bio-Gel P-2. Glycopeptides were then fractionated on concanavalin A–Sepharose. A column (0.7cm × 5cm) of concanavalin A–Sepharose (10mg of concanavalin A/ml of packed gel) was washed with 200ml of 20mM-Tris buffer, pH7.5, containing 100mM-NaCl, 1mM-$MgCl_2$, 1mM-$MnCl_2$, and 1mM-$CaCl_2$. Glycopeptides were applied and the column was washed with 25ml of buffer to remove tri- and tetra-antennary complex chains. The column was then eluted with 25ml of 10mM-α-methyl glucoside in buffer to remove biantennary complex chains, and then with 25ml of 500mM-α-methyl mannoside in buffer to elute the high-mannose structures. Fractions (1ml each) were collected and a sample from each was removed for determination of radioactivity.

Glycopeptides and oligosaccharides were also separated by gel filtration on a 1.5cm × 200cm column of Bio-Gel P-4. Columns were equilibrated and run in 0.7% acetic acid. These columns were standardized with various oligosaccharides. H.p.l.c. of the oligosaccharides was done on a Beckman Model 344 h.p.l.c. apparatus with an

Econophere silica μM Amino-bonded column (0.46cm × 25cm) from Alltech Associates. The apparatus was equipped with a precolumn disposable cartridge (0.46cm × 3cm) from Varian Associates. Oligosaccharides were eluted with acetonitrile/water (13:7, v/v) at a flow rate of 1.5ml/min. Fractions (40 drops) were collected and a sample of each fraction was measured for radioactivity.

Enzymic and chemical methods

Various enzymic treatments were done as previously described (Pan *et al.*, 1983). Briefly, Pronase digestions were done in 50mM-Tris buffer, pH7.5, containing 1mM-$CaCl_2$ and a few drops of toluene. Pronase solution (1ml, 5mg/ml) was added and another 1ml was added after 24h. Digestion with Endo H was done in 50mM-citrate buffer, pH6.0, with 5munits of enzyme and a few drops of toluene. Another 5munits of enzyme were added after 18h (Tarentino & Maley, 1974). Digestions with jack-bean α-mannosidase were performed in 100mM-sodium acetate buffer, pH5.0, containing 0.4mM-$ZnCl_2$. One unit of enzyme was added initially and another unit after 18h. In each case, the products resulting from these digestions were identified by chromatography on columns of Bio-Gel P-4.

Complete acid hydrolysis of oligosaccharides or glycopeptides was done in 2M-trifluoroacetic acid at 110°C for 4h (Akhrem *et al.*, 1979). Monosaccharides were identified by paper chromatography. Oligosaccharides were subjected to complete methylation as described by Hakamori (1964). After complete acid hydrolysis of the methylated oligosaccharides, the methylated sugars were identified by t.l.c. on silica-gel plates in benzene/acetone/water/NH_3 3000:4000:20:27 (by vol.) (Chapman *et al.*, 1980).

Results and discussion

Alterations in concanavalin A binding caused by castanospermine

Since previous studies showed that castanospermine inhibited the processing of the influenza-virus haemagglutinin and led to the accumulation of $Glc_3Man_7GlcNAc_2$ structures (Pan *et al.*, 1983), it was of interest to test this alkaloid in a non-viral system. Thus we examined glycoprotein synthesis in a mouse lymphoma cell line (BW 5147) and a mutant ($PHA^R2.7$) that lacks the processing glucosidase II.

Glycopeptides were prepared from normal and mutant lymphoma cells grown in the presence and absence of castanospermine and labelled with [2-^{3}H]mannose. The glycopeptides were subjected to concanavalin A–Sepharose affinity chromatography as shown in Fig. 1. Figs. 1(*a*) and 1(*b*) compare the parent-cell glycopeptides and demonstrate the alterations induced by this alkaloid. Thus, in the absence of alkaloid, about 29% of the radioactivity emerged in the wash, indicating it was present in the tri- and tetra-antennary complex chains, 12% was eluted with 10mM-α-methyl glucoside, indicating the presence of biantennary complex structures, and the remaining 59% required 500mM-α-methyl mannoside for elution, showing that this radioactivity was in high-mannose oligosaccharides. Fig. 1(*b*) shows that when the parent was grown in castanospermine, only 7.9% of the radioactivity was in the wash, 9.5% was eluted with 10mM-α-methyl glucoside and over 80% was in the 500mM-α-methyl mannoside-eluted fraction. This shift in the distribution of radioactivity from tri- and tetra-antennary complex chains to the high-mannose structures increased with increasing amounts of castanospermine in the medium. Thus, in the absence of alkaloid, 59% of the [^{3}H]mannose was in high-mannose structures, and this increased to 73% at a castanospermine concentration of 25μg/ml, to 80% at 50μg/ml, and to 82% at 100μg/ml. Since an alkaloid concentration of 100μg/ml caused the most dramatic effect, this concentration was used throughout these studies.

Figs. 1(*c*) and 1(*d*) show the elution patterns of the mutant cell line grown in the presence and absence of castanospermine. In this case, the alkaloid has relatively little effect, only changing the amount of radioactivity in the high-mannose structures from 77.2% (in the absence of alkaloid; Fig. 1*c*) to 83.6% in the presence of alkaloid. These data are not surprising, since the mutant lacks glucosidase II and produces mostly $Glc_2Man_{7-9}GlcNAc_2$ structures (Reitman *et al.*, 1982). However, the oligosaccharides in the mutant cells grown in castanospermine were different from those in the absence of alkaloid.

The lymphoma cells were also labelled with [^{3}H]galactose in the presence and absence of castanospermine. These glycopeptides were examined on concanavalin A columns as shown in Table 1. It can be seen that most of the radioactivity incorporated into polymer in either the presence or absence of alkaloid emerged in the wash. Since a considerable amount of the galactose label in the glucosidase II mutant also was found in the wash, it seems likely that most of this label is in proteoglycans. Nevertheless, the data in Table 1 clearly indicate that castanospermine had a marked effect on galactose incorporation into *N*-linked oligosaccharides and greatly increased the amount of label in high-mannose-structures. This increase was also seen with the glucosidase II mutant. These results are in keeping with the formation of glucose-containing high-mannose oligosaccharides

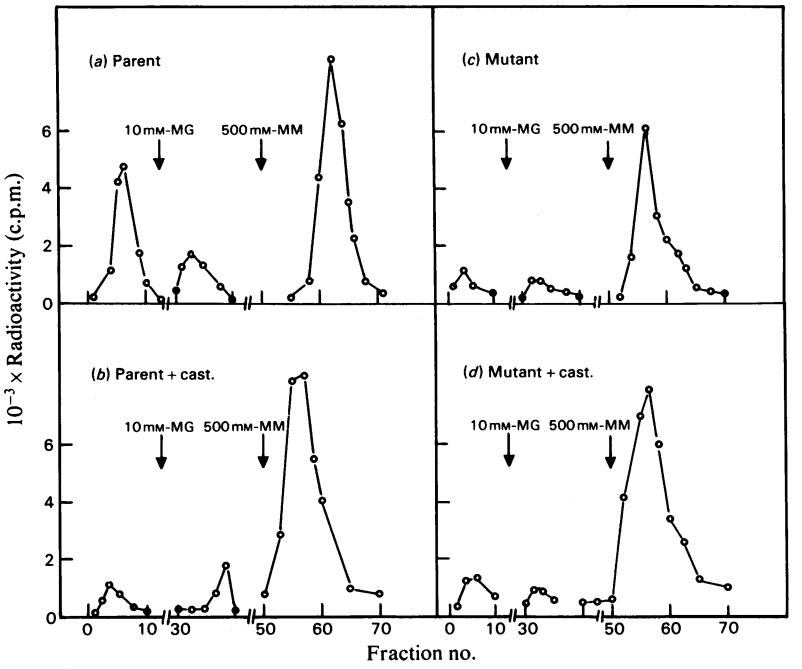

Fig. 1. *Effect of castanospermine (cast.) on the oligosaccharide composition of mouse lymphoma glycoproteins*
Mouse lymphoma parent cells and a mutant cell line lacking glucosidase II were grown in the presence of 100 μg of castanospermine/ml or in the absence of the alkaloid. Glycoproteins were labelled by the addition of [2-^{3}H]mannose to the medium. The glycopeptides were isolated from the Pronase-digested cells and run on columns (0.7 cm × 5 cm) of concanavalin A–Sepharose. The unbound glycopeptides were washed from the column with a buffer consisting of 20 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl, 1 mM-$MgCl_2$, 1 mM-$MnCl_2$ and 1 mM-$CaCl_2$. Loosely bound glycopeptides were eluted from the column with 10 mM-α-methyl glucoside (MG) in the same buffer, and tightly bound glycopeptides were eluted with 500 mM-α-methyl mannoside (MM) in the same buffer. A portion of each fraction was removed for the determination of radioactivity. (*a*) Elution pattern of glycopeptides from parent cells; (*b*) that of parent cells grown in castanospermine; (*c*) that of glycopeptides from mutant cells; (*d*) that of mutant cells grown in castanospermine.

Table 1. *Radioactive galactose in various fractions eluted from concanavalin A–Sepharose columns*
Values in parentheses refer to the percentage of total radioactivity in that fraction.

Cell lines and conditions	Radioactivity (c.p.m.) in: Wash	10 mM-α-Methyl glucoside	500 mM-α-Methyl mannoside
Wild-type	35200 (88.3)	3550 (8.9)	1160 (2.7)
Wild-type plus castanospermine	14900 (60.1)	2100 (8.4)	7800 (31.4)
Mutant line	17800 (67.1)	3400 (12.8)	5300 (20)
Mutant line plus castanospermine	6700 (31.1)	3950 (18.3)	10900 (50.5)

($Glc_3Man_{7-9}GlcNAc_2$) in the presence of castanospermine.

Analysis of oligosaccharides by gel filtration

In order to examine the effect of castanospermine on oligosaccharide structure, the mannose-labelled and galactose-labelled glycopeptides eluted from the concanavalin A columns with 500 mM-α-methyl mannoside were chromatographed on Bio-Gel P-4 columns as shown in Fig. 2. The glycopeptides were then digested with Endo H and rechromatographed on the Bio-Gel P-4 columns.

Fig. 2 shows the profiles of the [^{3}H]galactose-labelled glycopeptides. Fig. 2(*a*) demonstrates that the parent-cell glycopeptides were resolved into two radioactive peaks emerging before and after the $Man_9GlcNAc$ standard. As seen in Fig. 2(*b*),

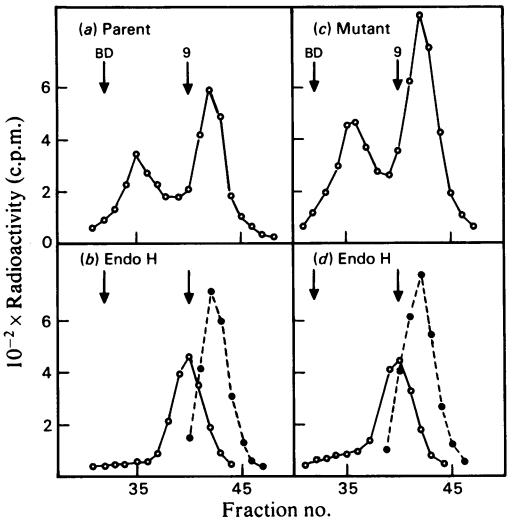

Fig. 2. *Effect of castanospermine on the oligosaccharide composition of the mouse lymphoma cellular glycoproteins*
Parent and mutant cells labelled with [1-^{3}H]galactose were grown in castanospermine (100μg/ml). The glycopeptides that bound to the concanavalin A–Sepharose column, after passage through a Bio-Gel P-2 column, were chromatographed on a column (1.5cm × 150cm) of Bio-Gel P-4 (*a* and *c*). The lower profiles (*b* and *d*) show these glycopeptides after digestion with Endo H. Abbreviations: BD, Blue Dextran; 9, $Hex_9GlcNAc$.

the first peak was susceptible to Endo H, and the released oligosaccharide emerged just before the $Man_9GlcNAc$ standard, indicating it was probably a $Hex_{10}GlcNAc$, or larger. On the other hand, the second peak in Fig. 2(*a*) was resistant to Endo H and no shift in migration occurred. On the basis of its migration it was smaller in size than the $Man_9GlcNAc$, but since it still contained an undetermined number of amino acid residues, the exact size of the carbohydrate could not be determined. Since both of these peaks were labelled with [^{3}H]galactose, it seemed likely that they contained glucose. In fact, complete acid hydrolysis followed by paper chromatography demonstrated that more than 90% of the radioactivity was present in glucose. Fig. 2 shows that the mutant-cell glycopeptides produced in the presence of castanospermine were quite similar to those of the parent cells (compare Figs. 2*a*–2*c*). Also, after treatment with Endo H, the mutant-cell glycopeptides and oligosaccharides were almost identical with those of the parent cell (compare Figs. 2*b* and 2*d*).

Identification of castanospermine-induced oligosaccharides by h.p.l.c.

For additional characterization, the oligosaccharides released by Endo H were sized by h.p.l.c., as shown in Fig. 3. Fig. 3(*a*) shows the profile of the mannose-labelled oligosaccharides from parent cells grown in castanospermine. The major radioactive oligosaccharides were sized as $Hex_{10}GlcNAc$ and $Hex_{11}GlcNAc$, with a small amount of $Hex_{12}GlcNAc$. However, in the galactose-labelled oligosaccharide from the parent cell (Fig. 3*b*), the major oligosaccharide was the $Hex_{10}GlcNAc$ and the next major peak was the $Hex_9GlcNAc$, with lesser amounts of $Hex_{11}GlcNAc$. Since the galactose experiment was done at a different time and with sets of cells different from those used in the mannose experiments, these results may reflect differences in the state of the cells, or in the growth cycle. They may also reflect differences in the pool sizes of intermediates, such as sugar nucleotides, that are involved in incorporation of mannose and galactose into glycoproteins.

Figs. 3(*c*) and 3(*d*) show the oligosaccharides obtained from the mutant cells in the presence of castanospermine. In both the mannose-labelled (Fig. 3*c*) and the galactose-labelled (Fig. 3*d*) oligosaccharides, the major species was a $Hex_{10}GlcNAc$, but substantial amounts of $Hex_9GlcNAc$ and $Hex_{11}GlcNAc$ were also observed. As indicated previously, most of the radioactivity in the galactose-labelled oligosaccharides was actually in glucose.

The oligosaccharides were treated with various enzymes to aid in their characterization. Table 2 shows that the mannose-labelled $Hex_{10}GlcNAc$ and $Hex_{11}GlcNAc$ from parent cells grown in castanospermine were only partially susceptible to α-mannosidase, indicating that they contained blocking glucose residues. This is in keeping with the notion that castanospermine inhibits the processing glucosidases. In addition, the $Hex_8GlcNAc$ to $Hex_{11}GlcNAc$ from mutant cells raised in the presence or absence of castanospermine were only partially susceptible to α-mannosidase. On the other hand, the $Hex_8GlcNAc$ produced in castanospermine-grown parent cells was very susceptible to α-mannosidase and yielded a ManGlcNAc and free mannose. The presence of this oligosaccharide may indicate that castanospermine at 100μg/ml is not sufficient to inhibit all processing completely. In this regard, the high-mannose structures produced by parent cells in the absence of alkaloid are also completely susceptible to α-mannosidase. It should also be mentioned that an Endo H-resistant, high-mannose structure produced in the presence of castanospermine (see Fig. 2) was also only partially susceptible to α-mannosidase, and some 14% of the radioactive mannose was released.

The galactose-labelled oligosaccharides ($Hex_{10}GlcNAc$ and $Hex_{11}GlcNAc$) were incubated with a particulate extract from lymphoma cells to deter-

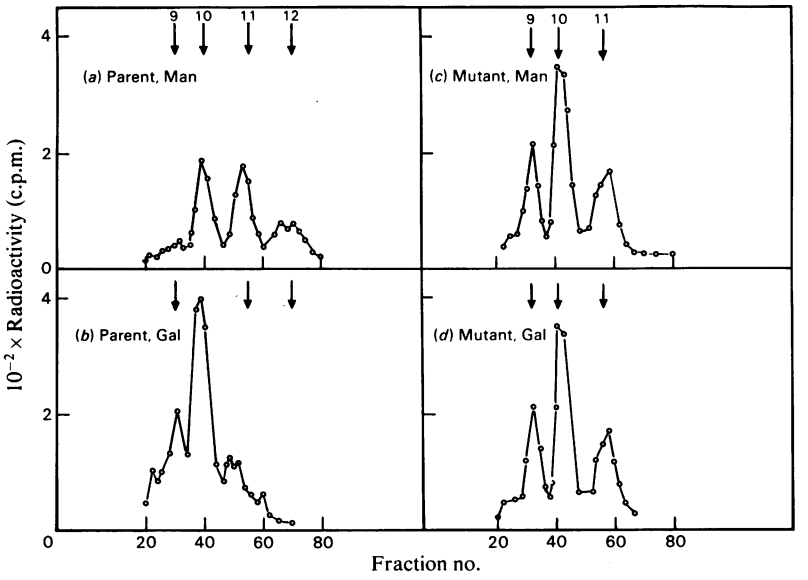

Fig. 3. *Molecular sizing of the oligosaccharides induced by castanospermine in parent and mutant cell lines*
The oligosaccharides from the [^{3}H]mannose-labelled and [^{3}H]galactose-labelled cells grown in the presence of alkaloid (100 μg/ml) were isolated on Bio-Gel P-4 after release by Endo H. These oligosaccharides were sized by h.p.l.c. (*a*) shows the h.p.l.c. pattern of parent-cell oligosaccharides labelled with [^{3}H]mannose (Man), whereas (*b*) shows that of parent cells labelled with [^{3}H]galactose (Gal). Profile (*c*) is the h.p.l.c. pattern of mutant-cell oligosaccharides from [^{3}H]mannose-labelled cells, whereas profile (*d*) is from [^{3}H]galactose-grown cells. Standards shown by arrows are: 9, $Hex_9GlcNAc$; 10, $Hex_{10}GlcNAc$ etc.

Table 2. *Products of α-mannosidase treatment of endoglycosidase H-released oligosaccharides*

	Radioactivity in:		
	Oligosaccharide	Man-β-GlcNAc	Mannose
Oligosaccharide source	(c.p.m.) (%)	(c.p.m.) (%)	(c.p.m.) (%)
Parent BW 5147 (control)	None	1572 (20.0)	6255 (80.0)
Parent BW 5147 (castanospermine)			
$Hexose_{11}GlcNAc$	4950 (70)	None	2125 (30.0)
$Hexose_{10}GlcNAc$	3262 (79.5)	None	844 (20.5)
$Hexose_8GlcNAc$	1495 (13.8)	1270 (11.7)	8010 (74.3)
Mutant PHA^R2.7 (control)			
$Hexose_{10}GlcNAc$	2889 (60.0)	195 (4.7)	1101 (26.3)
$Hexose_9GlcNAc$	3465 (70)	95 (4.7)	1395 (28.1)
$Hexose_8GlcNAc$	2181 (70.2)	Traces	924 (29.1)
Mutant PHA^R2.7 (castanospermine)			
$Hexose_{11}GlcNAc$	2685 (68.1)	112 (2.9)	1148 (29.1)
$Hexose_{10}GlcNAc$	3951 (63.0)	None	2322 (37.0)
$Hexose_9GlcNAc$	1677 (63.0)	None	981 (37.0)

mine whether this extract could release [^{3}H]glucose. About 50–75% of the radioactivity in the parent or mutant oligosaccharides was released as free glucose by this enzyme treatment. Of interest here is the finding that most of this activity could be inhibited by the adddition of castanospermine to these extracts (see also Table 3).

Methylation analysis of castanospermine-induced oligosaccharides

The castanospermine-induced oligosaccharides from parent and mutant cells were subjected to complete methylation to identify the glycosidic linkages and to determine the number of glucose

residues contained in each oligosaccharide. Fig. 4 presents results from thin-layer plates of the methylated mannose derivatives from the parent [^{3}H]mannose-labelled $Hex_{12}GlcNAc$ (left profile) and the mutant [^{3}H]mannose-labelled Hex_{10}-GlcNAc (right profile). The parent oligosaccharide showed the presence of 2,4-dimethylmannose (i.e. 3,6-linked), 3,4,6-trimethylmannose (2-linked), 2,4,6-trimethylmannose (3-linked) and 2,3,4,6-tetramethylmannose (terminal) in the approximate ratio of 1.8:4.5:1.0:1.8. This is close to the expected ratio of 2:4:1:2 for a $Glc_3Man_9GlcNAc_2$ structure. Also, the $Hex_{10}GlcNAc$ from the mutant cell showed the presence of 3,6-linked, 2-linked, 3-linked, and terminal mannose in a ratio of 1.7:1.7:1.0:1.8. This is close to the expected ratio of 2:2:1:2 for a $Glc_3Man_7GlcNAc$.

In order to show that the oligosaccharides did contain three glucose residues, the [^{3}H]galactose-labelled oligosaccharides were also subjected to methylation, and the methylated glucose derivatives were analysed by t.l.c., as shown in Fig. 5. The profile shown in Fig. 5(*a*) is from the parent cell oligosaccharide, whereas that in Fig. 5(*b*) is from the mutant cells. In both cases, three methylated glucose derivatives were observed, corresponding to 2,3,4,6-tetramethylglucose (i.e. terminal), 2,4,6-trimethylglucose (3-linked) and 3,4,6-trimethylglucose (2-linked). In the parent cell, these three derivatives were present in the approximate ratio of 1:1:1, but in the mutant there was a higher-than-expected amount of terminal glucose. In spite of that discrepancy, the results do indicate that castanospermine-induced oligosaccharides contain three glucose residues.

Analysis of Endo H-resistant oligosaccharides by methylation

In parent and mutant cells grown in castanospermine, an Endo H-resistant glycopeptide that sized like a $Hex_8GlcNAc_2$ was observed. Although this glycopeptide was also detected in cells grown in the absence of alkaloid, it was much more prevalent in the presence of castanospermine. This glycopeptide, labelled with either [^{3}H]mannose or [^{3}H]-galactose, was subjected to methylation and the methylated sugars were subjected to t.l.c., as shown in Fig. 6. The mannose-labelled material (Fig. 6*a*) gave rise to 3,6-linked, 2-linked, 3-linked, and terminal mannose in the approximate ratio of 1.1:1.6:1.1:1.0. In addition, the glycopeptide also contained glucose, but surprisingly, only two methylated glucoses were observed, corresponding to terminal glucose and 2-linked glucose. These two derivatives were present in almost equal amounts. These data suggest that the structure of the oligosaccharide portion of this glycopeptide is Glc(1,2)Glc(1,3)Man(1,2)Man(1,2)Man(1,3)-[Man(1,6)]ManGlcNAcGlcNAc.

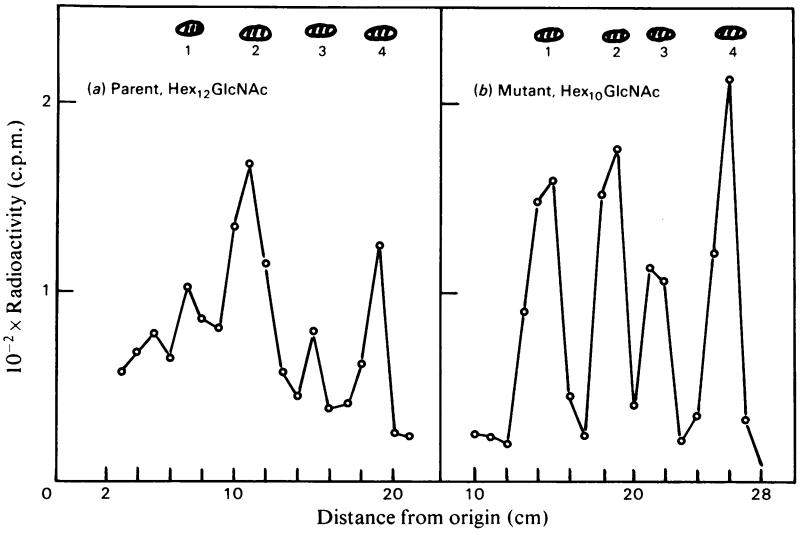

Fig. 4. *Methylation analysis of the [^{3}H]mannose-labelled oligosaccharides induced by castanospermine in parent (a) and mutant (b) cells*

In (*b*) the [^{3}H]mannose-labelled $Hex_{10}GlcNAc$ oligosaccharide from mutant cells was subjected to complete methylation, and after acid hydrolysis the methylated sugars were analysed by t.l.c. In (*a*) the [^{3}H]mannose-labelled $Hex_{12}GlcNAc$ from parent cells was methylated. Standard methylated sugars were as follows: 1, 2,4-dimethylmannose; 2, 3,4,6-trimethylmannose; 3, 2,4,6-trimethylmannose; 4, 2,3,4,6-tetramethylmannose.

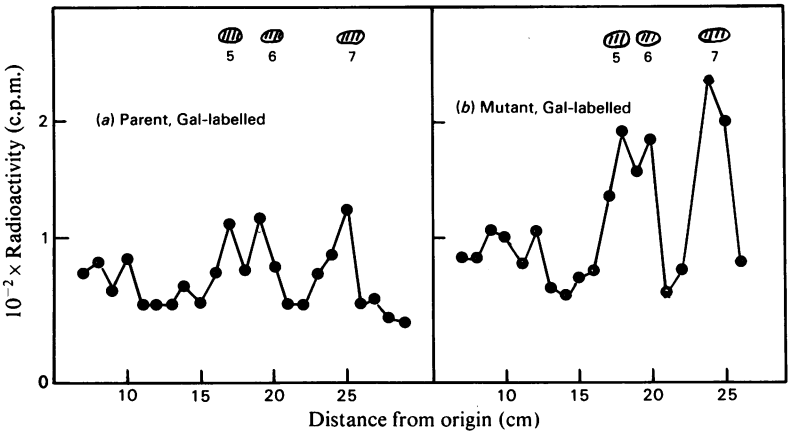

Fig. 5. *Methylation analysis of* [^{3}*H*]*glucose-containing oligosaccharides induced by castanospermine in parent* (*a*) *and mutant* (*b*) *lymphoma cells*

The [^{3}H]glucose-labelled $Hex_{10}GlcNAc$ [derived from [^{3}H]galactose(Gal)-grown cells] from the Bio-Gel P-4 columns were subjected to complete methylation, and after acid hydrolysis the methylated sugars were identified by t.l.c. Standard sugars were as follows: 5, 3,4,6-trimethylglucose; 6, 2,4,6-trimethylglucose; and 7, 2,3,4,6-tetramethylglucose.

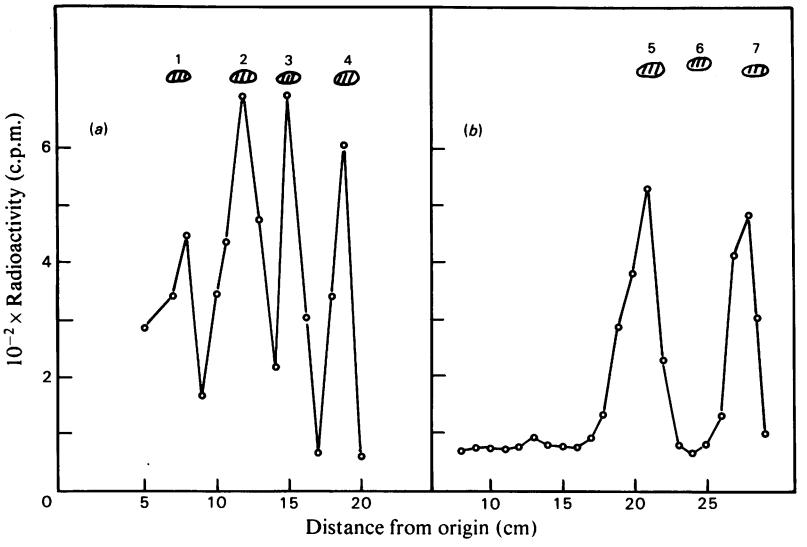

Fig. 6. *Methylation analysis of the Endo H-resistant oligosaccharides*

The castanospermine-induced glycopeptides that bound to concanavalin A but were resistant to Endo H digestion were subjected to methylation. After acid hydrolysis the methylated sugars were identified by t.l.c. Profile (*a*) is that of the [^{3}H]mannose-labelled oligosaccharide, whereas that in (*b*) is that of the [^{3}H]galactose-labelled oligosaccharide. Standards were as shown in Figs. 4 and 5.

Inhibition of glucosidase I and glucosidase II by castanospermine

In order to determine whether castanospermine inhibited glucosidase I and glucosidase II, an extract of the parent or mutant lymphoma cells was incubated with either [^{3}H]galactose-labelled $Glc_3Man_9GlcNAc$ or $Glc_2Man_9GlcNAc$ to measure the presence of the above enzymes. At the end of the incubation, the reaction was stopped by heating, and then hexokinase and ATP were added to convert any liberated glucose into glucose 6-phosphate. The amount of radioactivity that bound to, and was eluted from, a column of

Dowex-1 (Cl^- form) was measured. Table 3 shows that the extract from parent lymphoma cells contained both glucosidase I and glucosidase II, since radioactive glucose was released from both $Glc_3Man_9GlcNAc$ and $Glc_2Man_9GlcNAc$. The mutant cell line, on the other hand, contained glucosidase I, but glucosidase II was not detectable. Table 3 also shows that castanospermine inhibited both glucosidase I and glucosidase II in the parent-lymphoma-cell extract. It also inhibited glucosidase I in the mutant cell line. From these studies and from inhibition curves obtained by plotting castanospermine concentration against glucosidase activity, it appears that glucosidase I is more sensitive to this inhibitor than is glucosidase II. Nevertheless, both processing glucosidases are inhibited by castanospermine.

The present results show that castanospermine caused the accumulation of glucose-containing Endo-H-sensitive oligosaccharides that were characterized as $Glc_3Man_{7-9}GlcNAc$ in parent lymphoma cells. Castanospermine also caused a slight alteration in the oligosaccharide structure of the mutant lymphoma cells, consistent with the formation of a $Glc_3Man_7GlcNAc$ structure. These data indicated that castanospermine was inhibiting glucosidase I, and experiments *in vitro* with extracts of lymphoma cells also indicated that castanospermine was an inhibitor of glucosidase I and II.

In addition to inducing the formation of the above oligosaccharides, castanospermine also caused the formation of unusual oligosaccharides in these lymphoma cells. Thus, in both the parent and mutant cell lines, an Endo H-resistant oligosaccharide was observed that was either not present, or present in very low concentrations, in the absence of inhibitor. This structure appeared to migrate near the $Man_8GlcNAc_2$ standard, although it could not be sized exactly because it contained an undetermined number of amino acids. Methylation studies indicated that this compound was a Glc(1,2)Glc(1,3)Man(1,2)Man(1,2)-Man(1,3)[Man(1,6)]ManGlcNAcGlcNAc. $Man_5GlcNAc_2$ kinds of structures have been found in cells starved for glucose (Rearick *et al.*, 1981; Turco, 1980). These workers postulate an alternative pathway that branches off during synthesis of the lipid-linked saccharides at the dolichyl pyrophosphate $Man_5GlcNAc_2$ stage [see Hubbard & Ivatt (1981) or Elbein (1979) for reviews]. This intermediate is then presumably glucosylated to form dolichyl-pyrophosphate $Glc_3Man_5GlcNAc_2$, which can serve as the donor of oligosaccharide to protein. It is possible that castanospermine causes a situation in these cells that is analogous to glucose starvation, perhaps by inhibiting the uptake of glucose. However, such oligosaccharides were not found in influenza-virus-infected MDCK cells in the presence of this alkaloid (Pan *et al.*, 1983).

Similar kinds of Endo H-resistant oligosaccharides were isolated from Thy^{-1} mouse lymphoma cells that lack the ability to produce dolichyl phosphate mannose (Kornfeld *et al.*, 1979; Chapman *et al.*, 1980). Dolichyl phosphate mannose is the mannosyl donor for mannose units 6, 7, 8 and 9 of the lipid-linked saccharides. Thus these mutant cells can only make dolichyl pyrophosphate $Man_5GlcNAc_2$ and glucosylate this intermediate. It has also been found that cells treated with carbonyl cyanide *m*-chlorophenylhydrazone, an inhibitor of oxidative phosphorylation, do not form dolichyl phosphate mannose, although dolichyl phosphate glucose is still produced (Datema & Schwarz, 1981). Such cells also

Table 3. *Inhibition of glucosidase I and glucosidase II by castanospermine*

Lymphoma cells were ruptured by homogenization and the particulate material was isolated by centrifugation and suspended in buffer as the source of enzyme. Incubation mixtures contained 1200 c.p.m. of substrate (Glc_3- or Glc_2-$Man_9GlcNAc$), cell-free extract of the indicated cells (about 1 mg of protein), 100 mM-phosphate buffer, pH 7.0, containing 0.1% Nonidet P-40, and 50 μg of castanospermine/ml as indicated. Incubations were for 1 h at 37°C. Reactions were stopped by heating, and then hexokinase (1 unit) and ATP (5 μmol) were added to convert any liberated glucose into glucose 6-phosphate. The mixtures were passed through Dowex-1 (Cl^- form), and, after thorough washing with water, the glucose 6-phosphate was eluted and counted for radioactivity. The values in parentheses in the last column refer to the percentage of the original radioactivity released as glucose 6-phosphate.

Substrate used	Enzyme source	Castanospermine	Radioactivity in glucose 6-phosphate (c.p.m.)
$Glc_3Man_9GlcNAc$	BW 5147	Absent	360 (30)
$Glc_3Man_9GlcNAc$	BW 5147	Present	168 (12)
$Glc_3Man_9GlcNAc$	PHA^R 2.7	Absent	437 (37)
$Glc_3Man_9GlcNAc$	PHA^R 2.7	Present	106 (9)
$Glc_2Man_9GlcNAc$	BW 5147	Absent	677 (58)
$Glc_2Man_9GlcNAc$	BW 5147	Present	346 (30)

produce similar types of Endo H-resistant oligosaccharides. However, the Endo H-resistant oligosaccharides found in the lymphoma cells in the presence of castanospermine were presumably $Glc_2Man_5GlcNAc_2$ structures rather than the expected $Glc_3Man_5GlcNAc_2$. This is puzzling, since castanospermine inhibits glucosidase I and should prevent removal of the terminal glucose. This suggests that only two glucose residues were transferred to the dolichyl pyrophosphate $Man_5GlcNAc_2$. Also unexpected is the fact that the terminal glucose appeared to be 2-linked and the next glucose 3-linked. There is no precedent for this to our knowledge, but isolation and identification of the oligosaccharides attached to lipids of castanospermine-inhibited cells may clarify this question.

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