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Accumulation of pentamannose oligosaccharides in human mononuclear leukocytes by action of swainsonine, an inhibitor of glycoprotein processing †

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ABSTRACT

Swainsonine, a known inhibitor of the α-mannosidase II involved in processing of asparagine-linked glycoproteins, causes accumulation of hybrid-type oligosaccharide-containing glycoproteins in mammalian cells. Swainsonine augments lymphokine-activated, killer-cell induction at suboptimal doses of interlekin-2; the amount needed to increase LAK activity is 100–1000 fold higher than required to completely inhibit mannosidase II. Human mononuclear lymphocytes, when treated with these relatively high (58 μM) concentrations of swainsonine showed a 3–4 fold increase in D-[^{3}H]mannose incorporation into the glycan as compared to glycans of untreated cells. Analysis indicated accumulation of high-mannose type, free oligosaccharides in the soluble fractions of the cell. Chromatographic analysis of glycan obtained by D-[2-^{3}H]mannose labeling of human mononuclear lymphocytes showed synthesis of a new oligosaccharide, at 58 μM of swainsonine, that contained 36% of the total radioactivity incorporated into the glycan (oligosaccharide pool). This oligosaccharide fraction was resistant to hydrolysis by endoglycosidase H, endoglycosidase F, O and *N*-glycanase, but was susceptible to cleavage by Jack bean α-mannosidase and was bound > 90% to concanavalin A–Sepharose. A similar chromatographic elution profile was obtained from glycans labeled with D-[2-^{3}H]mannose from mouse $B_{16}F_{10}$ melanoma and baby hamster kidney cells subsequent to swainsonine treatment. Methylation analysis of free oligosaccharides obtained from MNL revealed the presence of a pentamannose. These results indicate the accumulation of a free high-mannose oligosaccharide rather than expected hybrid-type structure on treatment of cells with relatively high concentrations of swainsonine.

INTRODUCTION

Swainsonine, a plant toxin isolated from the Australian plant *Swainsona canescens*[1], from locoweed (*Astragalus lentiginosus*)[2], from a fungus (*Rhizoctonia legu-*

† Abbreviations: Swainsonine, SW; interleukin-2, IL-2; human mononuclear lymphocytes, MNL; lymphokine-activated killer, LAK; endo-β-*N*-acetylglucosaminidase H, Endo-H; baby hamster kidney, BHK; concanavalin A, Con A; *N*-acetylglucosamine, GlcNAc; glucose$_3$–mannose$_{7-9}$*N*-acetylglucosamine, G_3; glucose, Glc; mannose, Man.

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minicola)[3], is a potent inhibitor of lysosomal and Jack bean α-D-mannosidase[4,5]. Swainsonine is also a specific inhibitor of α-mannosidase II, an enzyme involved in the processing of asparagine-linked glycoproteins. In grazing animals, the ingestion of swainsonine-containing plants leads to a neurological condition, similar to α-mannosidosis (lysosomal storage disease) in humans. In the presence of swainsonine (0.03–0.06 μM), hybrid-type oligosaccharides of glycoproteins are synthesized with a 50% change from complex-type to hybrid-type structures[6–8]. Swainsonine inhibits lung colonization of $B_{16}F_{10}$ melanoma cells in mice[9]. Swainsonine, administered to immunodeficient mice, restores their capacity to produce antibodies, inhibits the growth of a sarcoma, and reduces lung metastasis by $B_{16}F_{10}$ melanoma cells. These results suggest that swainsonine has immunomodulating activities[10]. Recently, it has been shown that swainsonine enhances proliferation of murine natural killer cells, and protects murine bone marrow from radiation and the cytotoxic effects of chemotherapy[11–13]. At concentration 100–1000 fold higher than needed to inhibit α-mannosidase II in cell culture, swainsonine potentiates lymphokine-activated killer-cell induction at suboptimal doses of IL-2[14,15].

We have here investigated the effect of relatively high concentrations of swainsonine on glycoprotein biosynthesis by human mononuclear lymphocytes, and have compared these results with the effect of other inhibitors of α-mannosidase II. At these high concentrations (58 μM) of swainsonine, accumulation of unusual high-mannose oligosaccharide was observed. The significance of these findings in relationship to biological activity of swainsonine is discussed.

EXPERIMENTAL

Materials.—Endo-β-*N*-acetylglucosaminidase H (Endo-H), endoglycosidase F, O-glycanase, *N*-glycanase, deoxymannojirimycin, and chemically synthesized swainsonine (SW), were obtained from Genzyme (Boston, MA). A sample of SW isolated from *Astragalus lentiginosus* was a gift from Alan D. Elbein (University of Arkansas, Little Rock, AR). Manostatin A was a gift from Spencer Knapp (Rutgers State University, Newark, NJ). Castanospermine was isolated from *Castanospermum australe* seeds at the Marion Merrell Dow Research Institute[16]. D-[2-^{3}H]Mannose (specific activity, 20–30 Ci/mmol) and D-[1-^{3}H]Galactose (specific activity, 10–25 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Pronase was purchased from CalbioChem (San Diego, CA). Bio-Gel P-4 (200–400 mesh) was from BioRad Laboratories (Richmond, CA). Concanavalin A (10 mg/mL of packed gel), Blue dextran, stachyose, D-mannose, and α- and β-D-mannosidases were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]$Glc_3Man_{7-9}GlcNAc$ (G_3) was prepared as described earlier[17]. All other chemicals were from commercial sources and were of the highest purity available.

Baby hamster kidney (BHK) cells were a gift from Dr. Ken Yamada (National Institutes of Health, Bethesda, MD). $B_{16}F_{10}$ murine melanoma cells were propagated in C57/BL mice and were cultured in MEM (Gibco and Grand Island, NY),

supplemented with anti-PPLO, PSN antibiotic mixture and 10% fetal calf serum (FCS). BHK cells were grown as a monolayer in DMEM supplemented with 10% fetal calf serum, PSN antibiotic mixture, and L-glutamine. Human peripheral blood mononuclear leukocytes (MNL) were isolated by Ficoll–Hypaque density sedimentation. MNL (10^6 cells/mL) cell were cultured in MEM supplemented with 10% nonmitogenic FCS in 24-well tissue plates (Costar, Cambridge, MA). Inhibitors were added at the initiation of cell culture. Following an incubation period, usually of 72 h in a 37°C incubator with 5% CO_2, the cells were harvested, washed with PBS (3 ×) by centrifugation and analyzed as described next.

Labeling of oligosaccharides in the cells.—D-[2-^{3}H]Mannose-labeled glycans were prepared by metabolically labeling exponentially growing BHK or $B_{16}F_{10}$ melanoma cells. Cells were plated in 150-mm dishes (Falcon No. 1013) at a density of $2–5 \times 10^6$ cells/dish and allowed to grow. After 24 h, the media were replaced with 10–15 ml of fresh medium and inhibitors were added. After incubation of inhibitors with the cells, for 3 h label was added (10–20 μCi/mL of media) and incubation continued for 48 h for uniform labeling of the cells. MNL cells were labeled as described earlier[14]. At the end of the labeling period, the cells were washed (3 ×) with PBS and glycopeptides were prepared as described[7]. The cell pellet was heated for 10 min to 100°C to inactivate enzymes and centrifuged in a microfuge for 30 min to recover free oligosaccharides. Glycopeptides were prepared by exhaustive digestion of the cell pellet with Pronase under toluene saturated atmosphere for 72 h. In some cases, where lipid-linked oligosaccharides were prepared, heated cell pellets were centrifuged to remove free oligosaccharides and the pellets obtained by centrifugation were first extracted with 2:1 $CHCl_3$–MeOH and then with 1:1:0.3 $CHCl_3$–MeOH–H_2O to afford lipid-linked oligosaccharides. Finally, the oligosaccharides were released from lipid-linked oligosaccharides by mild acid hydrolysis[18].

Chromatographic procedures.—The glycopeptides and oligosaccharides were characterized by chromatography on Bio-Gel P-4 as described earlier[14]. Samples were applied to a column of Bio-Gel and eluted with 0.2% AcOH. The radioactivity in the fractions was determined by counting the fractions in universal cocktail (ICN) in a liquid-scintillation analyzer (Tri-Carb, 1900 CA, Packard).

Methylation analysis.—Methylation analysis was performed as described[19]. The sample, dried under N_2, was sonicated in Me_2SO for 30 min, dimethyl sulfinyl carbanion and MeI were then added, and the mixture sonicated again for 4–5 h. Methylation was terminated by the addition of water. The product was extracted with $CHCl_3$, dried, and hydrolyzed for 4 h at 120°C in 2 M CF_3CO_2H. The sample was then reduced with $NaBH_4$ and the desalted product applied to TLC plates for analysis.

Enzymatic digestions.—All incubations were conducted under a toluene saturated atmosphere at 37°C for various periods of time. To obtain the glycopeptides, the cell pellet was digested with 1% Pronase in 0.5–1 mL volume in 100 mM Tris (pH 7.5) containing 10 mM $CaCl_2$. Pronase was added every 24 h and the

incubation continued for 72 h[7]. For treatment with α-mannosidase, 1 unit of Jack bean enzyme was added to 0.5 mL of 100 mM NaOAc (pH 4.5), containing 1 mM $ZnCl_2$ and incubation was carried for 48 h with addition of 1 unit of enzyme after 24 h. Endo-H and O-glycanase digestions were performed in 100 mM citrate-phosphate buffer at pH 6.0 in a total volume of 0.5 mL. Usually 10–20 mU of enzyme were added every 24 h for a total of 72 h. Digestions by endoglycosidase were performed in 100 mM NaOAc buffer (pH 5.5) in 0.1 mL with 20 mU of enzyme added every 24 h for 48 h. *N*-Glycanase digestion was effected with 100 mM sodium phosphate buffer (pH 8.5) in 0.1 mL with 1 unit of enzyme added every 24 h for 48 h. At the end of each digestion, the mixture was heated in a boiling-water bath for 10 min and samples chromatographed on Bio-Gel columns for further analysis.

RESULTS AND DISCUSSION

MNL were labeled with D-[2-^{3}H]mannose for 72 h in the absence and presence of 58 μM of SW and their glycans and oligosaccharides (total cell lysate containing pronase-derived glycans and free oligosaccharides) chromatographed on Bio-Gel P-4. The elution profile (Fig. 1) showed, in the presence of 58 μM of SW, two additional peaks at fractions 45 (Peak I, Fig. 1) and 48. Although the peak at fraction 48 was variable and was observed in cells treated with lower concentrations of SW (ref 14, Fig. 4), or even occasionally in control experiments where SW treatment was omitted (data not shown), Peak I (Fig. 1) appeared only when MNL were treated with 58 μM of SW. The appearance of Peak I was observed in all experiments when the higher concentrations of inhibitor were used. These concentrations of SW are 100–1000 fold higher than needed for complete inhibition of α-mannosidase II (IC_{50} 0.03–0.06 μM, which causes the synthesis of hybrid-type glycans[6–8]). When the glycans and oligosaccharide pool obtained was treated with Endo-H (an enzyme that splits the bond between the di-*N*-acetylchitobiose component of oligosaccharides containing hybrid and high mannose-type structures[6,19]), no hydrolysis of the Peak I component (Fig. 1) was observed, even when isolated Peak I was treated with 1 unit/mL of Endo-H for 48 h. Similar results were obtained with the Thy-1 mutant, in which lipid-linked oligosaccharides having high-mannose oligosaccharides were resistant to hydrolysis by Endo-H[20]. The $B_{16}F_{10}$ murine melanoma cells were grown as a monolayer and labeled with D-[2-^{3}H]mannose in the presence of different concentrations of SW. The glycans and oligosaccharides obtained (Experimental) were chromatographed on a column of Biogel P-4. An elution profile similar to that obtained by labeling MNL was obtained (Fig. 2). At a concentration of 100 μM of SW, a significant amount of radioactivity in fraction 31 (corresponding to Peak I, Fig. 1) was observed. A similar peak of radioactivity from glycans and oligosaccharides obtained by D-[2-^{3}H]mannose labeling of BHK cells in the presence of 100 μM of SW was observed (data not shown). Thus, in three different cell types investigated, in amounts far

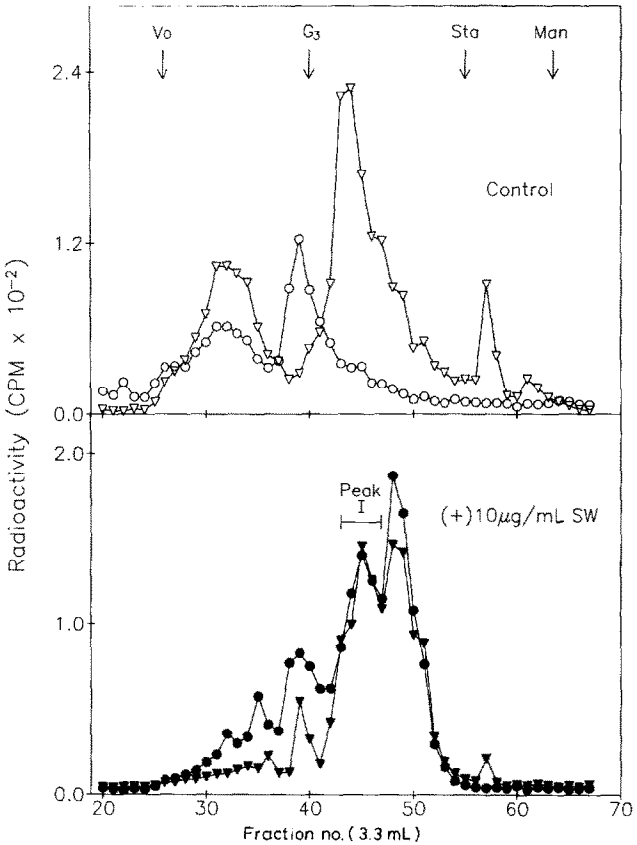


Fig. 1. Elution profile of MNL glycopeptides. MNL were labeled with D-[2-^{3}H]mannose in the absence or presence of 10 μg/mL (58 μM) SW for 72 h as described in the Experimental. Total glycopeptides prepared were chromatographed on Biogel P-4 (1.5×150 cm), before (circles) or after (triangles) treatment with Endo-H. Blue dextran (Vo), $G_3M_{7-9}N$ (G_3), stachyose (Sta) and mannose (Man).

exceeding the concentrations required to completely inhibit α-mannosidase II, a new oligosaccharide is generated. When SW obtained from different sources (chemically synthesized or isolated from *Astragalus lentiginiosus*) was used, similar results were obtained, indicating that a possible contaminant in the SW sample, or some of its isomers, is not responsible for accumulation of this oligosaccharide. Labeling of MNL cells with D-[^{3}H]mannose and D-[^{3}H]glucosamine gave similar oligosaccharide profiles. However, labeling with D-[^{3}H]galactose gave products devoid of label in the region of Peak I (data not shown), suggesting the absence of glucose in the oligosaccharides of Peak I.

Treatment of MNL with IL-2 (1–100 μ/mL) alone had no effect on the elution profile. However, when IL-2 (10 μ/mL) and SW (58 μM) were used, an elution profile similar to the one obtained with 58 μM SW (alone) was obtained, suggesting that IL-2, alone, or in combination with SW, had no effect on the accumulation of this oligosaccharide (data not shown). Treatment of MNL with

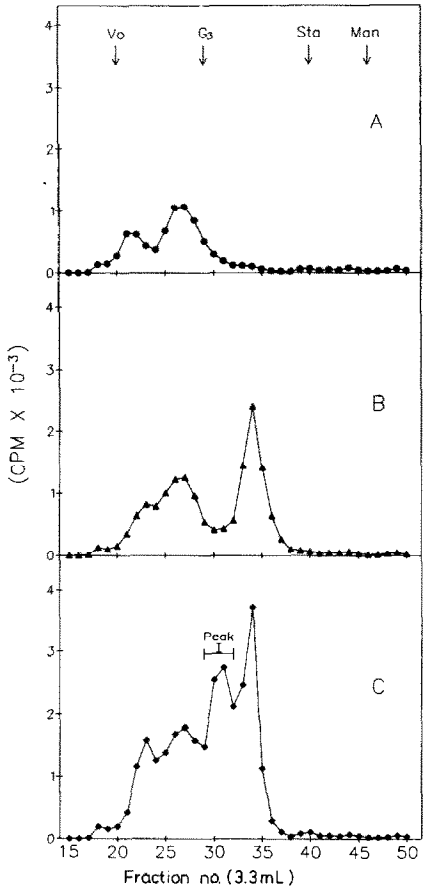


Fig. 2. Elution profile of $B_{16}F_{10}$ glycopeptides. $B_{16}F_{10}$ mouse melanoma were cells labeled with D-[2-^{3}H]mannose and total glycopeptides prepared as in Fig. 1 were chromatographed on Bio-Gel P-4 column (1.5×100 cm). A, cells were labeled in the absence of SW; B, cells were labeled in the presence of 0.173 μg/mL SW; C, cells were labeled in the presence of 17.3 μg/mL SW.

mannostatin A[21], a known inhibitor of α-mannosidase II and of lysosomal and intracellular α-mannosidases, resulted in accumulation of oligosaccharides comparable to the result for SW treatment (data not shown). However, treatment with such other inhibitors of glycoprotein processing as castanospermine (an inhibitor of α-glucosidase I), or deoxymannojirimycin (an inhibitor of α mannosidase I), did not cause accumulation of this oligosaccharide (data not shown). Thus, the accumulation of Peak I oligosaccharides occurs only with inhibitors of lysosomal and α-mannosidase II (SW and mannostatin) and at concentrations of SW that enhance LAK activity at suboptimal doses of IL-2.

Further analysis of the labeling of MNL with D-[2-^{3}H]mannose revealed that an unexpectedly high amount of radioactivity was incorporated into the total cell lysate of cells treated with 58 μM SW as compared to untreated control (Table I). In control samples, the amount of radioactivity recovered in supernatant fractions

TABLE I

Distribution of D-[^{3}H]mannose-labeled (cpm/10^6 cells) oligosaccharides in different fractions of cell lysates [a]

Treatment	Total lysate glycans and oligosaccharides	Supernatant oligosaccharides	$CHCl_3$–MeOH-extracted lipid-linked oligosaccharides	$CHCl_3$–MeOH glycans
Control	89000	53000	2142	62500
SW (10 μg/mL)	186000	183000	4285	50000

[a] PBL were labeled with D-[2-^{3}H]mannose for 72 h as described in the Experimental section. Cells were lysed by sonication and centrifuged at 100000*g* for 60 min at 4°C to recover the supernatant and the cell pellet. The cell pellet was extracted first with 2:1 $CHCl_3$–MeOH and then with 1:1:0.3 $CHCl_3$–MeOH–H_2O, and the combined $CHCl_3$–MeOH-extracted fractions were hydrolyzed to recover lipid-linked oligosaccharides. The cell pellet obtained after $CHCl_3$–MeOH extraction was digested with pronase to obtain the glycopeptide fraction (glycans).

and chloroform–methanol-extracted oligosaccharides was similar, with only a small amount of radioactivity recovered in the chloroform–methanol fractions. In cells treated with 58 μM SW, the amount of radioactivity in the chloroform–methanol extract and chloroform–methanol-extracted glycans did not change significantly as compared to the control. However, most of the increase in radioactivity in the total cell lysate with SW treatment occurred in the supernatant fraction (Table I). When D-[^{3}H]mannose-labeled oligosaccharides obtained from different fractions were chromatographed on columns of Bio-Gel P-4, no change in the elution profiles of oligosaccharides from the chloroform–methanol extract or glycans was observed (Fig. 3). However, when oligosaccharides from the supernatant fraction were chromatographed on the same column, a peak corresponding to Peak I of Fig. 1 was obtained in SW-treated samples. These data indicated that an unexpected increase of D-[^{3}H]mannose incorporation in the supernatant fraction could be attributed to oligosaccharides in Peak I. As these oligosaccharides are not obtained either from lipid-linked oligosaccharides or glycans, they presumably exist as free oligosaccharides in the cell.

Peak I oligosaccharides labeled with D-[^{3}H]mannose were pooled and treated with endoglycosidase F, O-, and *N*-glycanase. As expected, none of these enzyme hydrolyzed the oligosaccharides (as monitored by change in the elution profile on a column of Bio-Gel P-4, data not shown), suggesting the absence of asparagine-linked *N*,*N*-diacetylchitobiose. However, when Peak I oligosaccharides were treated with Jack bean α-mannosidase, 78% of the radioactivity in Peak I was released as mannose, and a peak of radioactivity near stachyose was obtained (Fig. 4), suggesting that 22% of the radioactive component resistant to α-mannosidase digestion was linked to a small oligosaccharide molecule. This remaining 22% of the radioactivity in the smaller peak was susceptible to the treatment of β-mannosidase with the release of most of the radioactivity in free mannose. When [^{3}H]GlcNAc-labeled oligosaccharides were used, treatment with α- and β-mannosidase resulted in a radioactive peak which migrated as free GlcNAc. However,

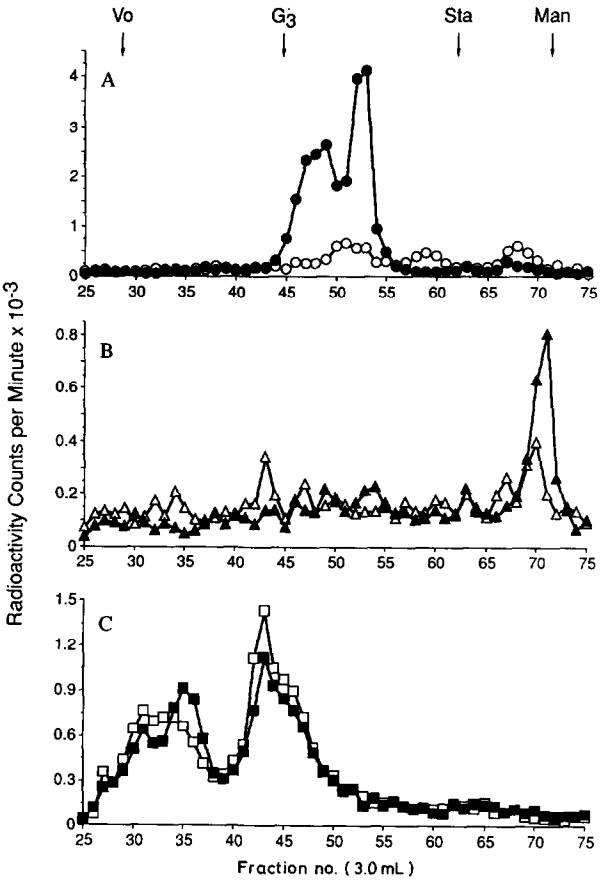


Fig. 3. Elution profile of different fractions of cell lysate. The fractions obtained in Table I were chromatographed on Bio-Gel P-4 (1.5×150 cm). Open symbols represent fractions for untreated control and filled symbols represent SW-treated fractions. A, supernatant fraction; B, $CHCl_3$–MeOH extract and acid released oligosaccharides; C, glycopeptides released by pronase treatment (glycans).

this peak was not susceptible to hydrolysis by β-*N*-acetylglucosaminidase, suggesting the absence of terminal GlcNAc on the oligosaccharide chain. These results suggest that Peak I oligosaccharides have α-linked mannose residues on the non-reducing end, and a final mannose residue was joined to GlcNAc by a β-linkage.

Further characterization of these oligosaccharide by methylation analysis (Fig. 5) suggested the formation of one (1.0) 2,4-di-*O*-methylmannosyl, two (1.7) 3,4,6-tri-*O*-methylmannosyl, and two (2.3) 2,3,4,6-tetra-*O*-methylmannosyl residues, suggesting the presence of a pentamannose linked to GlcNAc. The mannose β-linked to GlcNAc will give the 2,4-dimethyl ether. In this structure, the α-(→ 3)-linked mannose of the core trimannose is substituted with two α-(1 → 2)-linked mannoses. This structure is the same as that generated by amphomycin inhibition of synthesis of lipid-linked oligosaccharides[18] and in the Thy-1 mutant where synthe-

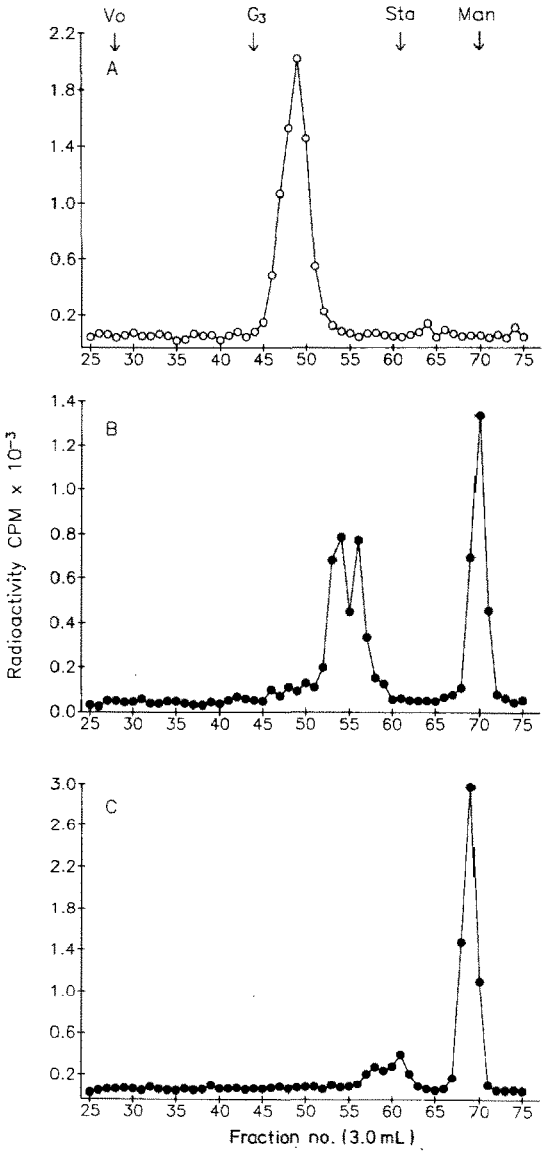


Fig. 4. Elution profile of Peak 1, Fig. 1 after treatment with Jack bean α-mannosidase. The products were chromatographed on Biogel P-4 (1.5 × 150 cm). A, No α-mannosidase; B, α-mannosidase digestion for 4 h; C, α-mannosidase digestion for 24 h.

sis of dolichyl phosphate mannose is blocked. In the Thy-1 mutant lymphoma cells of the class E complementation group, a similar methylation pattern was obtained[20]. Thus, in the presence of high concentrations of SW, free oligosaccharides having the pentamannose structure (structure **1**) accumulated in the cells in contrast to the hybrid-type oligosaccharides in the glycoproteins[6,7]. In the dolichyl phosphate cycle, these first five mannose residues are donated by GDP-mannose. It is at this

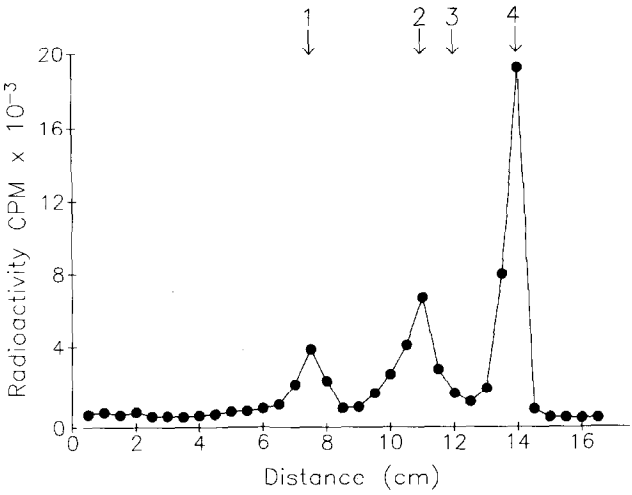
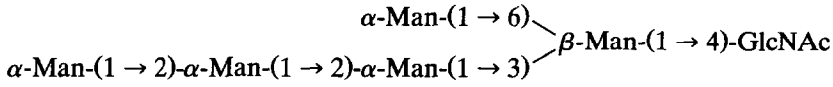


Fig. 5. Methylation analysis of MNL oligosaccharides. Peak I (Fig. 1) was pooled and methylated as described in the Experimental section. Methylation products were separated on silica gel plates in 50:200:3:1.5 benzene–acetone–H_2O–NH_4OH. The standards were (1) 2,4-di-*O*-methylmannose; (2) 3,4,6-tri-*O*-methylmannose; (3) 2,4,6-tri-*O*-methylmannose; and (4) 2,3,4,6-tetra-*O*-methylmannose.

step that additional mannose residues are donated by dolichyl mannosyl phosphate to the growing lipid-linked saccharide chain[22]. Thus, it is entirely possible that inhibition of Dol-P-Man synthesis or transfer by these higher concentrations of SW resulted in the accumulation of pentamannose oligosaccharides, which may cleave off the dolichol phosphate by hydrolytic enzymes of the cells to release free oligosaccharides. A further modification could be brought about by a hydrolase that cleaves the diacetylchitobiose of the released oligosaccharide.



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Interestingly in studies described here, the accumulation of high-mannose oligosaccharides resulted only when cells are treated with a compound that is a potent inhibitor of α-mannosidase II and of lysosomal α-mannosidase. This inhibitor has been demonstrated to modulate natural killer-cell activities. The significance of this co-relationship is not clear.

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