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Characterization of the high mannose asparagine-linked oligosaccharides synthesized by microfilariae of *Dirofilaria immitis*

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Abstract: This report describes the structures of high-mannose-type *N*-linked oligosaccharides in glycoproteins synthesized by the microfilariae of *Dirofilaria immitis*. Microfilariae of *D. immitis* were incubated *in vitro* in media containing 2-[^{3}H] mannose to allow metabolic radiolabeling of the oligosaccharide moieties of newly synthesized glycoproteins. Glycopeptides were prepared from the radiolabeled glycoproteins by digestion with pronase and fractionation by chromatography on concanavalin A-Sepharose. Thirty eight percent of 2-[^{3}H] mannose incorporated into the microfilariae of *D. immitis* glycopeptides was recovered in high mannose-type asparagine-linked oligosaccharides which were bound to the immobilized lectin. Upon treatment of 2-[^{3}H] mannose labeled glycopeptides with endo-β-*N*-acetylglucosaminidase H, the high mannose-type chains were released and their structures were determined by high performance liquid chromatography and exoglycosidase digestion. The major species of high mannose-type chains synthesized by microfilariae of *D. immitis* have the composition $Man_5GlcNAc_2$, $Man_6ClcNAc_2$, $Man_7GlcNAc_2$, and $Man_8GlcNAc_2$. Structural analyses indicate that these oligosaccharides are similar to high mannose-type chains synthesized by vertebrates.

Key words: *Dirofilaria immitis*, mannose, carbohydrates, oligosaccharides, microfilariae, metabolic labeling, HPLC, enzyme digestion

INTRODUCTION

Dirofilaria immitis is a mosquito-borne filarial nematode which causes heartworm disease. Heartworms typically inhibit the right ventricle, the pulmonary artery and pulmonary lobular arteries of dogs and other animals (Otto, 1975). Adults and microfilariae cause inflammation of the arterial wall and obstruction of blood vessels. Enlargement of the right side of the heart occurs with serious injury to the lungs, liver and kidneys. Heartworm infection in dogs usually results in serious chronic disease and occasionally in early death. Because the surface of a parasite interacts directly with body fluids and tissues of the host, an analysis of the surface composition of the parasite is of importance in elucidating its potential immunogenic function. Numerous studies have focused on the antigenic properties of the surface of parasitic nematodes (Jungery *et al.*, 1983).

Knowledge of the fine structure or cytochemistry of the filarial parasite surface would be useful in understanding the structural organization and the molecular biology of host-parasite interactions in filariasis. Recently, interest in carbohydrate antigenicity and composition of the parasite surface has increased considerably. The

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microfilariae of filarial parasites are either sheathed or unsheathed. Examples of sheathed microfilariae are *Brugia pahangi, B. malayi* and *Wuchereria bancrofti. Dirofilaria immitis, Onchocerca volvulus* and *O. lienalis* are examples of unsheathed microfilariae. Furman and Ash (1983a) suggested that sheath carbohydrate antigenic determinants may be the primary targets of the host's immune response. They also characterized the exposed carbohydrates on the sheath surface of *B. pahangi* microfilariae by lectin binding (Furman and Ash, 1983b). Previous studies have shown that the oligosaccharide moieties of glycoproteins are important in many aspects of cellular metabolism and immune recognition (Reading, 1984; Von Figura *et al.*, 1986).

Nyame *et al.* (1987, 1988a, 1988b) characterized the *N*-and *O*-linked oligosaccharides made by *Schistosoma mansoni.* In these papers, they demonstrated that the type of *O*-linked oligosaccharides made by the adult male *S. mansoni* is the unusual terminal *O*-linked *N*-acetylglucos-amine. Some of the *N*-linked oligosaccharides were found to be high mannose-type chains. The major species of high mannose-type chains synthesized by *S. mansoni* adult males have the composition $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$, which are similar to high mannose-type chains synthesized by mammalian cells.

An understanding of the structures and biosynthesis of glycoprotein oligosaccharides of the microfilariae of *D. immitis* might allow for a better understanding of the role of these molecules in the host/parasite relationship, particularly in terms of antigenicity of these molecules.

In this report, we describe the structures of the high mannose-type *N*-linked oligosaccharides synthesized by microfilariae of *D. immitis.*

MATERIALS AND METHODS

1. Materials

Concanavalin A-Sepharose (ConA-Sepharose) was obtained from Pharmacia P-L Biochemicals. α-methylmannoside, α-fucose, Sephadex G-25-80, Amberlite MB-3, Saponin were purchased from Sigma Chemical Co. Endo-β-*N*-acetylglucosaminidase H (Endo-H) was purchased from Boehringer Mannheim Biochemica. Materials for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were purchased from Bio-Rad Laboratories and NEN Research Products, respectively. Pronase was obtained from Calbiochem and Bio-Gel P6 was purchased from Bio-Rad laboratories. The 2-[^{3}H]mannose (21 Ci/mmol) was purchased from Amersham. The radioactive standards *N*-[^{3}H] acetylglucosamine and *N*-[^{14}C]acetylgalactosamine were prepared by mild acid hydrolysis of the radio-active sugar nucleotides in 10 mM HCl at 100°C for 10 minutes. The radioactive standards Man_{5-9}-$GlcNAc_1$ (Man, Mannose; GlcNAc, *N*-acetylglucosamine) were prepared from the mouse lymphoma cell line BW5147, after incubation of 2×10^6 cells in media containing 2-[^{3}H] mannose, as described previously (Cummings and Kornfeld, 1982). Endo H was purchased from Miles Scientific. Dulbecco' Modified Eagle's Medium (DMEM) was obtained from GIBCO.

2. Preparation of microfilariae

Microfilariae were recovered from the blood of dogs infected with *D. immitis* at TRS Labs Inc., Athens, Georgia. Microfilariae were isolated by a modification of the method of Sawyer and Weinsterin (1963). Microfilaremic blood was mixed (10:1) with 0.2% Saponin in 10 mM phosphate buffer saline (PBS) at pH 7. 4. This mixture was agitated in 10 seconds and diluted again (1:10) with PBS and stirred. This diluted mixture was filtered using a polycarbonate membrane filter with a pore size of 3.0 μm. After filtration, the filter was taken from filter holder and soaked in PBS at 37°C for 10 minutes. The filter was removed from the PBS solution, and the PBS solution containing microfilariae was centrifuged at 900 g for 10 minutes. The resulting pellet was washed twice with DMEM.

3. Metabolic radiolabeling of microfilariae with radioactive precursor sugars

Approximately 1×10^6 microfilariae were incubated with 1 mCi/ml of 2-[^{3}H] mannose in DMEM containing 10% fetal calf serum,

gentamicin (50 mg/ml), and nystatin (250 Unit/ml) in a final volume of 0.5 ml for 24 hours, to metabolically radiolabel their glycoprotein oligosaccharides. Incubations were performed at 37°C in a humidified incubator containing 5% CO_2. The microfilariae were motile and viable at the end of the incubation, and the culture was free of bacterial and fungal contamination.

4. Preparation of radiolabeled glycopeptides from microfilariae

Radiolabeled microfilariae were washed twice in DMEM and five times in PBS. They were sonicated in lysis buffer. The homogenate was extracted 3 times with 20 volumes of chloroform-methanol (2:1) to remove lipids. The residual protein was dried under a gentle stream of nitrogen and incubated with 10 mg/ml pronase in lysis buffer for 24 hr at 60°C in toluene atmosphere. After digestion with pronase, the sample was boiled for 5 minutes and desalted on a column (1 × 50 cm) of Sephadex G-25 in 7% 1-propanol. The radiolabeled glycopeptides, which were recovered in the void fractions, were pooled and dried in a shaker bath evaporator under vacuum.

5. Column chromatography

Radiolabeled glycopeptides were fractionated on 2-ml columns (0.7 × 5 cm) of Con A-Sepharose at room temperature (Cummings and Kornfeld, 1982). Glycopeptides bound to the column were eluted first with 10 mM α-methylglucoside followed by 100 mM α-methylmannoside(buffer at 60°C). Fractions of 2 ml were collected from the column and aliquots were taken and mixed with Scintiverse I (Fisher) for determining radioactivity in a liquid scintillation counter. Chromatography of glycopeptides and oligosaccharides on BioGel P6 was performed on a 1.5 × 100 cm column equilibrated in 0.1 M NH_4HCO_3 and 1 ml fractions were collected. Glycopeptides were desalted and separated from monosaccharides by chromatography on 1 × 50 cm columns of Sephadex G-25 in 7% 1-propanol.

6. Glycosidase treatments

Radiolabeled glycopeptides were treated with 10 mU of Endo H in 50 μl of 0.1 M citrate-phosphate buffer pH 5.6 at 37°C for 48 hours in a toluene atmosphere. Oligosaccharides released by Endo H treatment were separated by amine adsorption high-performance liquid chromatography (HPLC) on a Beckman Model 110 A dual pump system using a MicroPak AX-5 column (Varian) as described previously by Mellis and Baenziger (1981). The sample (20 μl) was injected and the elute was mixed with Scintiverse BD (Fisher) for the determination of radioactivity. Radiolabeled glycopeptides were treated with 10 mU of Endo H in 5 μl of 0.1 M citrate-phosphate buffer, pH 4.6 at 37°C for 48 hours in a toluene atmosphere.

After treatment with α-mannosidase, the samples were analyzed directly by descending paper chromatography in solvent III, ethyl acetate/pyridine/acetic acid/water (5:5:1:3).

RESULTS

Microfilariae were used to study the structures of glycoprotein oligosaccharides synthesized by *D. immitis* because a large amount of microfilariae could be separated using a new technique, which was developed to reduce the contamination of samples with blood cells and cell debris (Kang *et al.*, 1993).

They were metabolically radiolabeled *in vitro* with 2-[^{3}H] mannose to radiolabel glycoproteins synthesized by microfilariae of *D. immitis* and to allow subsequent analysis of the structure of the oligosaccharides moieties of the glycoproteins without contamination by host-derived glycoproteins.

1. Lectin affinity column chromatography of radiolabeled glycopeptides

Radiolabeled glycopeptides were prepared from the 2-[^{3}H] mannose-labeled glycoproteins by treatment with pronase and were then applied to columns of Con A-Sepharose (Fig.1). Unbound glycopeptides were eluted with buffer alone and were designated as I. Bound glycopeptides were first eluted with 10 mM α-methylglucoside, which resulted in the immediate elution of material designated as II. The remaining material on the column was eluted with 100 mM α-methylmannoside and

designated as III. Thirty eight percent of the 2-[^{3}H] mannose radioactivity recovered in glycopeptides was contained in III glycopeptides, which indicate that these glycopeptides contain substantial amounts of high mannose type oligosaccharides (Fig.1).

2. Analysis of the composition of radiolabeled III glycopeptides.

A portion of the 2-[^{3}H]mannose-labeled III glycopeptides was hydrolyzed and the released sugars were analyzed by descending paper chromatography. Ninety seven percent of the 2-[^{3}H] mannose radioactivity in III glycopeptides was recovered as mannose and the remaining 3% was fucose (Fig. 2). These results also show that the III glycopeptides consist of high mannose-type *N*-linked oligosaccharides.

3. Treatment of 2-[^{3}H]mannose-labeled III glycopeptides with Endo H

To further investigate whether III glycopeptides contained high mannose-type *N*-linked oligosaccharides, 2-[^{3}H] mannose-labeled III glycopeptides were treated with Endo H. Prior to Endo H treatment, III glycopeptides were applied to a column of Bio-Gel P6 (Fig.3). A majority of the radioactivity eluted in/or near the void volume of this column. The radioactive fractions were pooled and incubated with Endo H and then re-applied to the Bio-Gel P6 column. Endo H released about 94% of the radioactivity as marked by a shift of the radioactivity from the void or near the void volume to smaller-sized material after the enzyme treatment (Fig.3). This result suggests that III oligosaccharides contain high mannose-type *N*-linked oligosaccharides.

4. Digestion of 2-[^{3}H]mannose-labeled III glycopeptides with α-mannosidase

To confirm that III glycopeptides contained high mannose-type N-linked oligosaccharides, the III glycopeptides were treated with α-mannosidase and then analyzed by descending paper chromatography. α-Mannosidase released 96% of the total 2-[^{3}H]mannose-labeled radioactivity (Fig.4). This suggests that this oligosaccharide consists of high mannose-type *N*-linked oligosaccharides which are synthesized by the microfilariae.

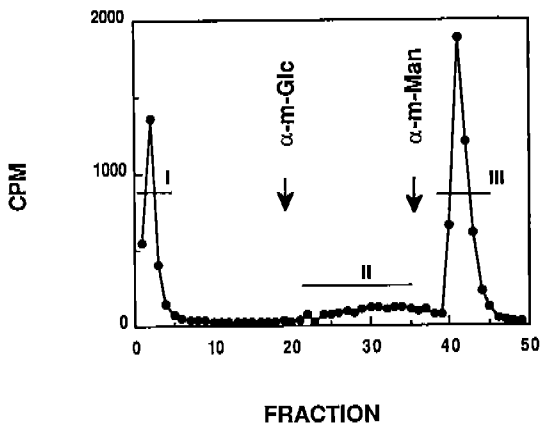

Fig. 1. Chromatography on Con A-Sepharose of radiolabeled glycopeptides. Radiolabeled microfilariae of *D. immitis* were homogenized and treated with Pronase to generate glycopeptides. The glycopeptides were applied to columns of Con A-Sepharose and fractions (2 ml) were collected. Bound glycopeptides were eluted first with 10 mM α-methylglucoside (α-m-Glc) followed by 100 mM α-methylmannoside (α-m-Man) as described in MATERIALS AND METHODS. The resulting glycopeptide fractions were pooled and designated I, II, III as indicated. Recovery of applied radioactivity was greater than 90% for column.

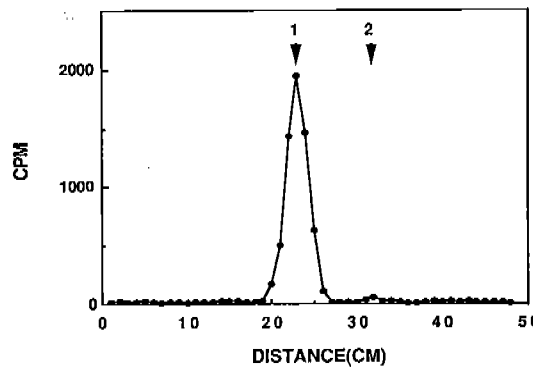

Fig. 2. Descending paper chromatogram of the 2-[^{3}H] mannose-labeled III glycopeptides after strong acid hydrolysis. An aliquot of the 2-[^{3}H] mannose-labeled III glycopeptides was hydrolyzed in 2 N HCl at 100°C for 4 hours and the released sugars were analyzed by descending paper chromatography as described in Materials and Methods. The migration positions of standards are indicated 1, mannose; 2, fucose.

5. HPLC of Endo H released oligosaccharides from 2-[^{3}H] mannose-labeled III glycopeptides.

The size of the Endo H released oligosaccharides (Fig. 3) were determined by HPLC. The Endo H released oligosaccharides were passed over a column of Amberlite MB-3 mixed bed resin to which they did not bind, indicating that the chains did not contain charged residues. These oligosaccharides were then separated by HPLC into four major peaks, which corresponded in elution to $Man_5GlcNAc_1$ (25%), $Man_6GlcNAc_1$ (19%), $Man_7GlcNAc_1$ (34%), and $Man_8GlcNAc_1$ (22%) (Fig.5).

Based on the susceptibility of the microfilariae of *D. immitis*-derived high mannose chains to Endo H, the sizes determined for the released oligosaccharides on HPLC and the results of exoglycosidase treatments, we propose that the high mannose-type chains have structures similar to those shown in Fig. 6. It is possible that there are several isomers of $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_5GlcNAc_2$.

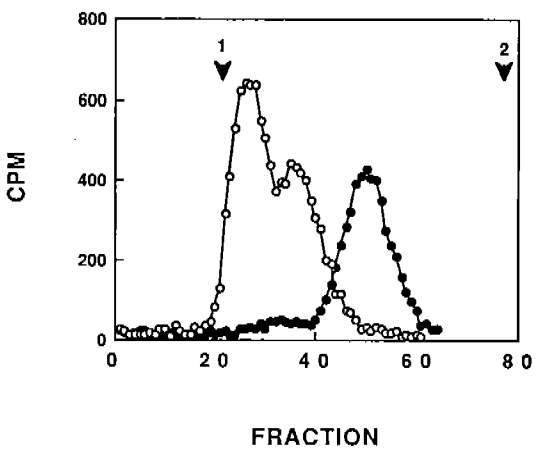

Fig. 3. Chromatography on Bio-Gel P6 of 2-[3H] mannose-labeled III glycopeptides before and after treatment with Endo H. 2-[^{3}H] mannose-labeled glycopeptides (from Fig. 1) were applied to a column of Bio-Gel P6. The glycopeptides were then pooled, desalted by column chromatography on Sephadex G25, treated with Endo H as described in Materials and Methods, and re-applied to the Bio-Gel P6 column. 1 and 2 shown were determined by elution position of bovine albumin and galactose respectively. (○) Untreated; (●) Endo H treated.

DISCUSSION

The technique of metabolic radiolabeling was employed in this study of the microfilariae of *D. immitis* glycoproteins because it allows the analysis of material synthesized by the parasite and prevents the possibility of contaminating analyses with host-derived glycoproteins. Previous studies have shown that 2-[^{3}H] mannose can biosynthetically radiolabel only mannose and fucose residues in animal cell glycoproteins (Kornfeld and Tabas, 1978). The amino sugars, *N*-acetylglucosamine, N-acetylgalactosmine,and sialic acid, can be biosynthetically radiolebeled by 6-[^{3}H] glucosamine (Cummings *et al.*, 1983). Galactose and glucose residues can be radiolabeled by 6-[^{3}H] galactose (Cummings and Kornfeld, 1984). Previous analyses by SDS-PAGE and fluorography of microfilariae of *D. immitis* metabolically labeled in vitro with these radioactive sugars, indicated that numerous microfilariae glycoproteins are labeled by only 2-[^{3}H] mannose and 6-[^{3}H] glucosamine precursors (Kang *et al.*, 1992). Moreover, Nyame *et al.* (1987, 1988, 1988a, 1988b) found that adult male schistosomes synthesize glycoproteins containing high

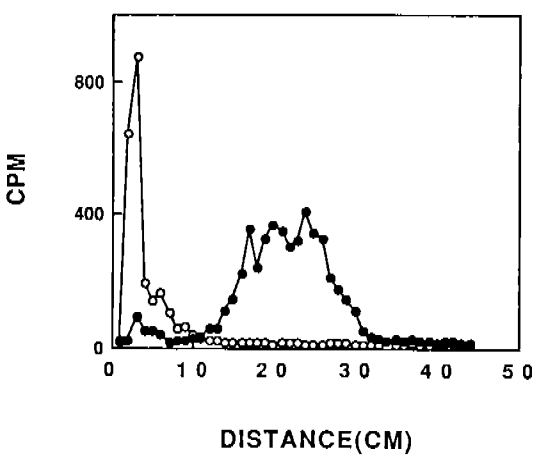

Fig. 4. Descending paper chromatogram of the 2-[^{3}H] mannose-labeled III glycopeptides after α-mannosidase treatment. After treatment with α-mannosidase, the samples were analyzed directly by descending paper chromatography in solvent III (○) Untreated; (●) Treated.

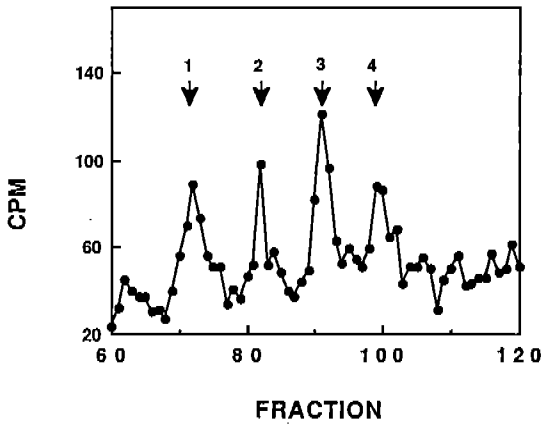

Fig. 5. Separation by HPLC of the 2-[^{3}H] mannose-labeled oligosaccharides released by Endo H treatment. The elution position of standards are indicated as 1, $Man_5GlcNAc_1$; 2, $Man_6GlcNAc_1$; 3, $Man_7GlcNAc_1$; 4, $Man_8GlcNAc_1$.

mannose-type asparagine-linked oligosaccharides that range in size from $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$. These oligosaccharides are similar in structure to the high mannose-type *N*-linked chains synthesized by mammalian cells. Our studies demonstrate that the microfilariae of *D. immitis* synthesize glycoproteins containing high mannose-type asparagine-linked oligosaccharides which range in size from $Man_5GlcNAc_2$ to $Man_8GlcNAc_2$. These oligosaccharides are similar in structure to the high mannose-type *N*-linked chains synthesized by many vertebrates.

Lectin affinity column chromatography, treatment with Endo H, digestion with α-mannosidase, and HPLC provide means of direct analysis of the high mannose-type oligosaccharides synthesized by a helminthic parasite. Previous studies in lectin affinity column chromatography (Cummings and Kornfeld, 1982) have demonstrated that Con A-Sepharose interacts with very low affinity with either most *O*-linked oligosaccharide or complex-type tri- and tetraantennary and bisected biantennary *N*-linked oligosaccharides. However, Con A-Sepharose interacts with relatively high affinity with many of the complex-type non-bisected biantennary N-linked oligosaccharides (Cummings and Kornfeld, 1982) and these may be contained in pool II. Glycopeptides III may consist of high mannose and/or hybrid-type *N*-linked oligosaccharides as well as ungalactosylated and unsialylated complex-type biantennary *N*-linked chains, as these types of structure are known to have the highest affinity for Con A-Sepharose (Cummings and Kornfeld, 1982).

Endo H cleaves between the two *N*-acetylglucosamine residues in the *N,N'*-di-*N*-acetylchitobiose core of certain high mannose and hybrid-type *N*-linked oligosaccharides. Successful cleavage results in the release of *N*-acetylglucosamine at the reducing termini from GlcNAc-Asn-R (Tai *et al.*, 1977). Using this method, we demonstrated high a cleavage rate and identified high mannose-type oligosaccharides.

It has been shown in studies of mammalian cell-derived oligosaccharides that high mannose-type chains contain outer α-linked mannose residues linked to a core mannose residue which is attached in β-1,4 linkage to the residue of N-acetylglucosamine (Li *et al.*, 1978). α-Mannosidase cleaves the four α1,2-linked mannose residues of the deglucosylated $Man_9GlcNAc_2$ intermediate to yield the $Man_5GlcNAc_2$ intermediate, and it cleaves the outermost α1,3- and α1,6-linked mannose residues of the $GlcNAc_1Man_5GlcNAc_2$ intermediate to yield $GlcNAc_1Man_3GlcNAc_2$ (Tabas and Kornfeld, 1982). This method also confirmed the presence of high mannose-type oligosaccharides synthesized by the microfilariae of *D. immitis*.

The pathway of *N*-glycosylation of proteins in mammalian, avian, insect, and fungal cells involves the transfer of a $Glc_3Man_9GlcNAc_2$ precursor from a dolichol pyrophosphate carrier to an asparagine residue of the nascent polypeptide in the endoplasmic reticulum (Kornfeld and Kornfeld, 1985). In mammalian cells the precursor is processed to high mannose-type chains of various sizes and complex-type *N*-linked chains (Kornfeld and Kornfeld., 1985). In contrast, many lower organisms are only capable of processing the precursor to high mannose-type chains. In yeast cells, the precursor is processed to $Man_8GlcNAc_2$ and then modified by addition of mannose residues to yeast mannans (Byrd *et al.*, 1982). Mosquito cells process the precursor

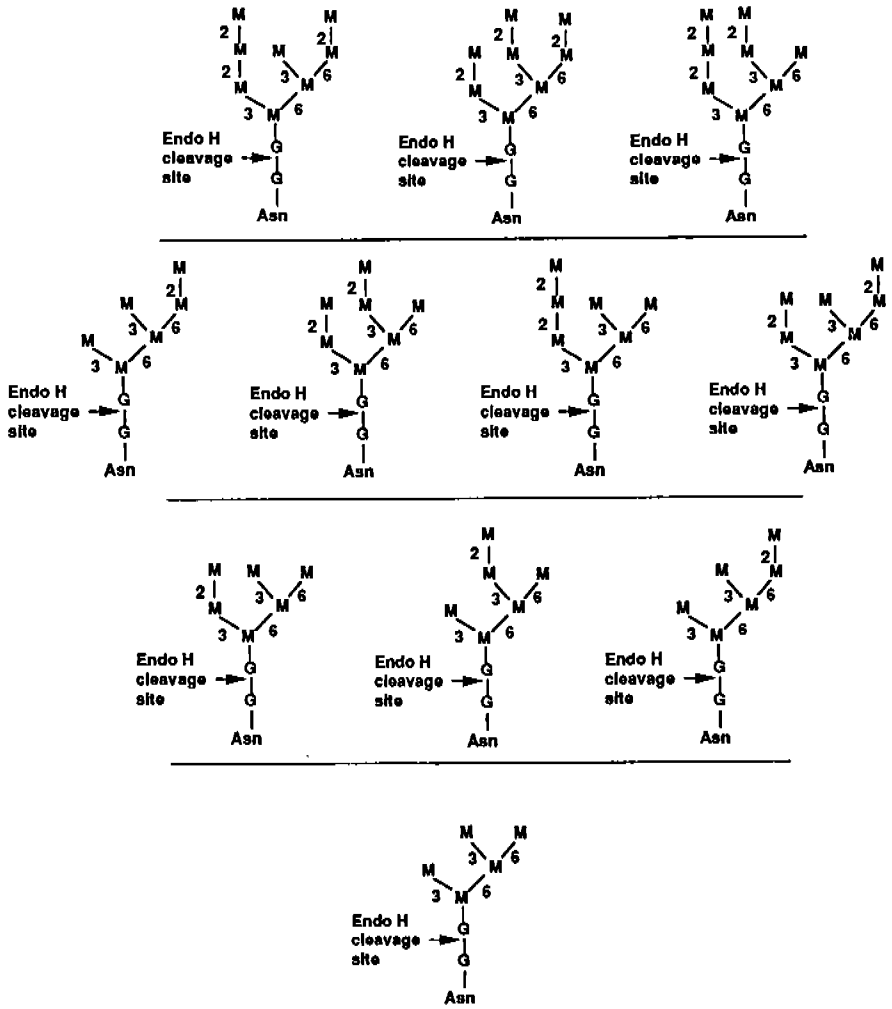

Fig. 6. Possible structures proposed for the high mannose-type *N*-linked oligosaccharides containing 5, 6, 7 and 8 mannose residues. M, mannose; G, *N*-acetylglucosamine; Asn, asparagine.

to a $Man_3GlcNAc_2$ structure, but they are unable to form complex-type *N*-linked oligosaccharides (Hsieh and Robbins., 1984). In the parasitic protozoa *Leishmania mexicana*, the dolichol pyrophosphate oligosaccharide precursor contains and undergo any further processing after transfer to protein (Parodi and Martin-Barrientos, 1984).

The processing pathway in microfilariae appears to be similar to that in vertebrates. We observed that many 2-[^{3}H]mannose-labeled glycopeptides derived from microfilariae of *D. immitis* glycoproteins are bound by Con A-Sepharose, suggesting that microfilariae may process high mannose-type chains rather than complex-type species.

The function of the high mannose-type chains in schistosome-derived glycoproteins are not known. In mammalian cells, phosphorylated high mannose chains occur on lysosomal enzymes and these function in the targeting of the enzymes to lysozymes (Von Figura and Hasilik, 1986). In addition, the high mannose-type chains are bound with high affinity by mannose receptors present in various cells, including macrophages (Tietze *et al.*, 1982), which allows for uptake and clearance of glycoproteins from body fluids

containing these side chains. Norden and Strand (1984) suggested that high mannose-type chains might occur on numerous immunogenic schistosome glycoproteins. The biological role(s) and fate of microfilariae of *D. immitis*-derived glycoproteins containing high mannose-type chains remain to be investigated. Also the subcellular location of these high mannose chains in microfilariae of *D. immitis* chains is not yet known.

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=국문초록=

심장사상충 자충이 합성한 high mannose asparagine-linked oligosaccharides의 분자화학적 분석

농촌진흥청 가축위생연구소 기생충과

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심장사상충 자충이 합성한 당단백의 분지(分枝) 역할을 하는 N-linked high mannose 타입 올리고당의 구조에 대한 조사를 수행하였다. 사상충 자충을 방사선 표식 2-[^{3}H] mannose를 함유한 배지에서 24시간 배양하였다. 자충으로부터 분리 정제한 당단백을 pronase로 소화시킨 다음, concanavalin A-Sepharose 크로마토그라피하여 분획하였다. 37%의 mannose가 자충 대사에 이용되었으며 Lectin chromatography를 사용하여 high mannose 타입의 올리고당을 회수하였다. 이 올리고당을 endo-β-N-acetylglucosaminidase H 효소로 소화시킨 후 HPLC를 사용하여 high mannose 타입 올리고당의 구성을 분석하였다. 심장사상충 자충이 합성한 high mannose 타입 올리고당의 형태는 $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$과 $Man_8GlcNAc_2$로 확인되었다. 이와 같이 심장사상충 자충이 체내 합성한 high mannose 타입 올리고당이 일반 척추동물이 체내 합성한 high mannose 타입 올리고당과 구조적으로 일치하였다.

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