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Changes in glycan branching and sialylation of the Thy-1 antigen during normal differentiation of mouse T-lymphocytes

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The glycans of the Thy-1 antigen present on thymocytes and lymph-node T-lymphocytes were investigated after external labelling of the cells. Neuraminidase, endoglycosidase H and endoglycosidase F were used in combination with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and isoelectric focusing in order to characterize the nature of the glycans on ^{125}I-labelled and immunoprecipitated Thy-1. Glycopeptides were prepared from Thy-1 obtained from cells labelled by periodate/boro[^{3}H]hydride treatment. The glycopeptides were separated by affinity chromatography on concanavalin A–Sepharose and analysed by gel filtration. The results show that both types of cells possess Thy-1 molecules with three *N*-linked carbohydrate chains, of which one is of 'high-mannose' type and the other two of triantennary and biantennary 'complex' type. The ratio of triantennary/biantennary chains was decreased on Thy-1 of mature cells compared with that of immature cells, but instead more sialic acid was present on these chains. Deglycosylated Thy-1 appeared to be of the same size regardless of origin, indicating that only the carbohydrate moiety differs between Thy-1 molecules of the two cell types.

The Thy-1 differentiation antigen is a membrane-bound glycoprotein present on the surfaces of mouse thymocytes/T-lymphocytes, neuronal cells and some other cell types (Campbell *et al.*, 1981). Since Thy-1 seems to be the most abundant glycoprotein on thymocytes (approximately 1×10^6 molecules per cell) (Carlsson & Stigbrand, 1983*a*) and contains a high proportion of carbohydrate (31%, w/w) (Carlsson & Stigbrand, 1983*b*), this molecule is proposed to carry a significant part of the total carbohydrate exposed on the surfaces of these cells. Carbohydrate structures on cell surfaces have been suggested to be involved in recognition events during development, growth and migration of cells (reviewed in Sharon & Lis, 1982). Structural studies of the oligosaccharide chains of major constituents on cell surfaces will facilitate the understanding of such phenomena.

Mouse thymocyte Thy-1 was previously shown to be glycosylated by three *N*-linked carbohydrate chains (Williams & Gagnon, 1982; Carlsson & Stigbrand, 1984). At one site (Asn-23) a 'high-mannose' chain, containing either five or six mannose residues, was identified, whereas the other two sites (Asn-75 and Asn-99) were glycosylated with 'complex-type' chains (Carlsson & Stigbrand, 1984). Both these sites were shown to be variably glycosylated with biantennary chains, with or without fucose, and with triantennary chains. Also, the sialylation of the 'complex-type' chains has been shown to vary (Carlsson & Stigbrand, 1982). Altogether, the variation in glycosylation of this molecule makes it highly heterogeneous, as clearly seen when assayed in different systems (Carlsson & Stigbrand, 1983*a*).

It was previously observed that the glycosylation of Thy-1 alters when thymocytes differentiate to immunocompetent T-lymphocytes (Carlsson & Stigbrand, 1983*a*). Thus T-lymphocyte Thy-1 was shown to be more acidic when assayed by isoelectric focusing, and the behaviour on lentil-lectin–Sepharose columns together with the appearance on SDS/polyacrylamide-gel electrophoretograms indicated that the molecule was less heterogeneous after maturation of the cells. Since alterations in the carbohydrate structure of Thy-1 during differentiation of T-lymphocytes may be of functional significance, the differences in glycosylation between thymocytes and peripheral T-

Abbreviation used: SDS, sodium dodecyl sulphate.

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lymphocytes were further characterized in the present study.

Materials and methods

Materials

NaB^3H_4 (350mCi/mmol), $Na^{125}I$ (17Ci/mg) and endoglycosidase F (*Flavobacterium meningosepticum*) were obtained from New England Nuclear. Concanavalin A–Sepharose (10mg/ml of gel), Pharmalyte 3-10 and Sepharose 4B were from Pharmacia Fine Chemicals. Lactoperoxidase and glucose oxidase (grade II) were from Boehringer Mannheim. Neuraminidase (*Clostridium perfringens*, type X) was from Sigma Chemical Co., endoglycosidase H (*Streptomyces plicatus*) was from Miles Laboratories, Pronase-CB (*Streptomyces griseus*) was from Calbiochem, Bio-Gel P-6 (200–400 mesh) was from Bio-Rad Laboratories and sodium metaperiodate was from Riedel–de Haen A.G. Mice of strain C3H were bred in the animal colony at the Department of Immunology, University of Umeå. Phosphate-buffered saline, pH7.2, contained 137mM-NaCl, 3mM-KCl, 8mM-Na_2HPO_4 and 1.5mM-KH_2PO_4.

Labelling of cells

Thymi and lymph nodes (axillary, brachial, inguinal and mesenteric) were dissected from mice of strain C3H. Cells were prepared and washed five times in phosphate-buffered saline by centrifugation, and surface-labelled by lactoperoxidase/glucose oxidase-catalysed iodination of tyrosine and by periodate/boro[^{3}H]hydride tritiation of sialic acid (Gahmberg & Andersson, 1977). Cells to be iodinated were suspended at 100×10^6 cells/ml in 1ml of 20mM-glucose/phosphate-buffered saline, to which was added 50μg of lactoperoxidase and 1mCi of $Na^{125}I$. The reaction was started by adding 10μg of glucose oxidase. After incubation for 30min at room temperature, the cells were diluted with 10ml of phosphate-buffered saline and layered over 1ml of foetal-calf serum, and centrifuged. One additional wash was performed, after which the cells were solubilized by adding 1ml of 2% (w/v) Nonidet P-40/1mM-phenylmethanesulphonyl fluoride/150mM-NaCl/50mM-Tris/HCl, pH8.0. After incubation on ice for 1h, non-solubilized material was removed by centrifugation at 100000*g* for 30min. Cells to be labelled by periodate/boro[^{3}H]hydride were suspended at 370×10^6 cells/ml (thymocytes) or 200×10^6 cells/ml (lymph-node cells) in 1ml of phosphate-buffered saline. Sodium metaperiodate was added to a final concentration of 2mM, and the cells were incubated at room temperature for 10min in the dark. The reaction was quenched by the addition of 100μl of 0.1M-glycerol, and the cells were washed twice with 50ml of phosphate-buffered saline and resuspended in 1ml of the same buffer. To the cell suspensions was added 1mCi of NaB^3H_4 (3μmol), and after incubation for 30min at room temperature the cells were washed twice with 50ml of phosphate-buffered saline. The cells were solubilized by the same procedure as described for iodinated cells except that twice the volume of solubilizing buffer was used.

Immunoprecipitation

Thy-1 was immunoprecipitated from cell lysates by use of rabbit anti-(purified mouse brain Thy-1) immunoglobulin (Carlsson & Stigbrand, 1983*a*) covalently coupled to Sepharose 4B (4mg of immunoglobulin/ml of wet gel). Settled beads (200μl) were added to 1ml of lysate (corresponding to 10^8 cells), and the suspension was incubated overnight at 4°C with slow agitation. The beads were washed by centrifugation three times with 2% Nonidet P-40/0.1% bovine serum albumin/phosphate-buffered saline and once in 0.1% Nonidet P-40/phosphate-buffered saline. Bound material was eluted by boiling for 5min in 600μl of 0.1% SDS per 200μl of beads. After removal of the supernatant the beads were washed with the same volume of 0.1% SDS and the supernatants were pooled.

The recovery of ^{125}I-labelled Thy-1 was 7×10^5c.p.m. and 3×10^5c.p.m. from 10^8 thymocytes and lymph-node cells respectively. For cells labelled with periodate/boro[^{3}H]hydride the recovery of [^{3}H]Thy-1 was approx. 10×10^3c.p.m. and 5×10^3c.p.m. from 10^8 thymocytes and lymph-node cells respectively. The efficiency in β-radiation counting was 40–50%.

Glycosidase digestions

All glycosidase digestions were performed for 24h at 37°C in a total volume of 50μl. Portions of ^{125}I-Thy-1 in 0.1% SDS were mixed with 5vol. of acetone and centrifuged. The precipitates were dissolved in 0.5% Nonidet P-40/0.5M-sodium acetate buffer, pH4.5, for digestion with 40munits of neuraminidase. For endoglycosidase H digestion, samples were dissolved in 0.1% SDS/0.1M-sodium phosphate buffer, pH6.0, and boiled for 3min before 1munit of endoglycosidase H was added. Samples to be treated with endoglycosidase F were dissolved in 0.1% SDS/0.5% Nonidet P-40/1% 2-mercaptoethanol/0.1M-sodium phosphate buffer, pH6.0, and boiled for 3min, after which 0.8 unit of endoglycosidase was added. The digestions were terminated by boiling for 3min, and the samples were finally precipitated with acetone and dissolved in buffer for analysis by polyacrylamide-gel electrophoresis and isoelectric focusing.

SDS/polyacrylamide-gel electrophoresis and isoelectric focusing

Polyacrylamide-gel electrophoresis in the presence of SDS was performed on 7.5–15% or 10–15%-polyacrylamide-gradient slab gels with the system described by Laemmli (1970). After electrophoresis the gels were stained with Coomassie Blue to locate molecular-mass markers ('low molecular weight' calibration kit; Pharmacia Fine Chemicals), dried and exposed to Kodak X-Omat AR X-ray film. For detection of tritiated samples the gel was treated with Amplify (Amersham) before being left to dry, and the X-ray film was exposed at −70°C.

Isoelectric focusing was performed on a 5%-polyacrylamide slab gel containing 6M-urea, 5% Pharmalyte 3-10 and 2% Nonidet P-40 as described previously (Carlsson & Stigbrand, 1982). Radioactive material was detected after drying and exposure to X-ray film.

Preparation and analysis of glycopeptides

Tritiated Thy-1, labelled in sialic acid residues with periodate/boro[^{3}H]hydride, was precipitated with acetone and dissolved in 0.5ml of 0.1M-Tris/HCl buffer, pH7.8, containing 1mM-$CaCl_2$. Pronase (25μg) was added, and the mixture was incubated for 48h at 60°C. Additional Pronase (25μg) was added after 24h. The digestion was terminated by boiling for 3min.

Analytical gel filtrations were performed on a 1cm × 100cm column of Bio-Gel P-6 (200–400 mesh) equilibrated with 0.1M-NH_4HCO_3. Samples of volume 1ml were applied and the flow rate was 6ml/h. Fractions (1ml) were collected, and portions (100 or 200μl) of each fraction were mixed with 2ml of Aquasol (New England Nuclear) and their radioactivities counted in a scintillation counter. The column was calibrated with bovine serum albumin (void-volume marker) and with [^{3}H]acetylated glycopeptides prepared from purified mouse thymocyte Thy-1 (Carlsson & Stigbrand, 1984). Free mannose was eluted at 80ml.

Glycopeptides with different glycan structures were separated on a 2ml concanavalin A–Sepharose column equilibrated with 5mM-sodium acetate buffer, pH5.2, containing 1mM-$CaCl_2$ and 1mM-$MnCl_2$. The samples were dissolved in the same buffer and applied with a flow rate of 6ml/h. The column was washed with 15ml of equilibration buffer, after which the bound material was eluted with 25mM-methyl α-D-mannoside in equilibration buffer. Fractions (1ml) were collected, and portions (50 or 100μl) were mixed with Aquasol and their radioactivities measured.

Pooled glycopeptide fractions were concentrated by freeze-drying. In both gel filtrations and concanavalin A chromatographies the yields were better than 80%.

Results

Analysis of Thy-1 by use of glycosidases

Thy-1 antigens from thymocytes and peripheral T-lymphocytes have previously been shown to give different band appearances on SDS/polyacrylamide-gel electrophoretograms (Carlsson & Stigbrand, 1983*a*). Thus thymocyte Thy-1 appears as a broad band ranging in apparent M_r from approx. 25000 to 30000. Sometimes several distinct bands can be seen within this broad band (Carlsson & Stigbrand, 1983*b*). On the basis of glycopeptide analysis, it was proposed that this heterogeneity was due to differences in the types of carbohydrate chains attached to the peptide (i.e. triantennary and biantennary 'complex-type' chains and 'high-mannose' chains) (Carlsson & Stigbrand, 1984). The highest-M_r forms would thus contain a higher proportion of triantennary chains. In contrast, mature T-lymphocyte Thy-1 appeared less heterogeneous on SDS/polyacrylamide-gel electrophoretograms, since the high-M_r species were missing. A 'high-mannose' chain was shown to be invariably present at one site (Asn-23) on thymocyte Thy-1 (Carlsson & Stigbrand, 1984), whereas the sialic acid content on the 'complex-type' chains was shown to vary (Carlsson & Stigbrand, 1982). In order to test which factors contribute to the differences in band appearance, immunoprecipitated ^{125}I-labelled Thy-1 from thymocytes and lymph-node cells were treated with various glycosidases and analysed by SDS/polyacrylamide-gel electrophoresis. The difference between intact Thy-1 from the two sources was confirmed (Fig. 1, lanes a and b). Digestion with neuraminidase (Fig. 1, lanes c and d) affected the bands very little, despite the fact that thymocyte Thy-1 has been shown to contain on the average three sialic acid residues per molecule (Carlsson & Stigbrand, 1983*b*). After treatment with endoglycosidase H (which cleaves 'high-mannose' chains between the innermost *N*-acetylglucosamine residues; Tai *et al.*, 1977) a decrease in molecular mass corresponding to one glycan was observed for both types of Thy-1 (Fig. 1, lanes e and f). Still, however, the difference in heterogeneity remained. Endoglycosidase F has been shown to cleave both 'high-mannose' and 'complex-type' chains on intact glycoproteins, probably between the core *N*-acetylglucosamine residues (Elder & Alexander, 1982). By use of this enzyme a complete removal of the carbohydrate chains on Thy-1 was obtained (Fig. 1, lanes g and h). With limited digestion, Thy-1 molecules containing no, one, two and three glycan chains were detectable (results

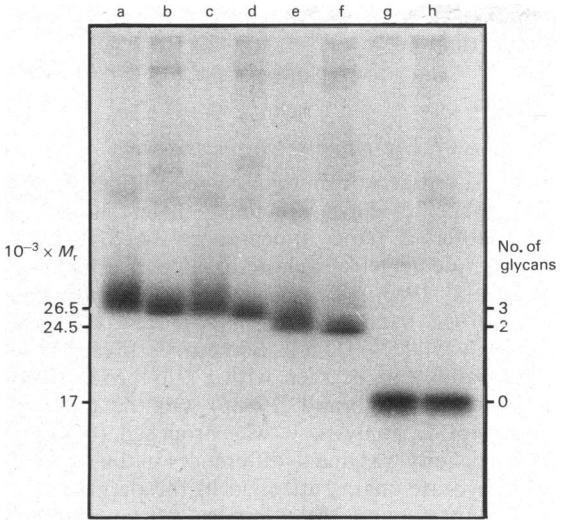

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of intact and glycosidase-treated ^{125}I-Thy-1*

Thy-1 was immunoprecipitated from surface-iodinated thymocytes (lanes a, c, e and g) and lymph-node cells (lanes b, d, f and h). Lanes a and b show intact Thy-1, lanes c and d neuraminidase-treated, lanes e and f endoglycosidase H-treated, and lanes g and h endoglycosidase F-treated Thy-1. For experimental details see the text.

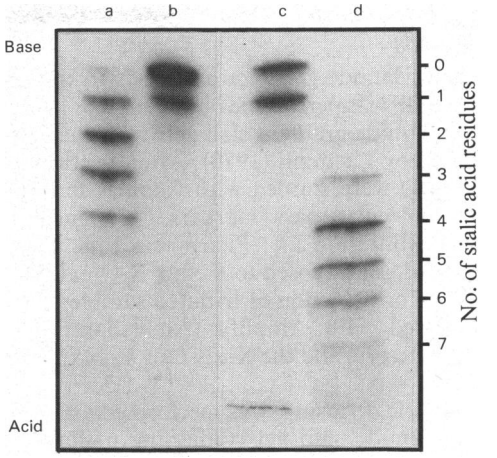

Fig. 2. *Isoelectric focusing of intact and neuraminidase-treated ^{125}I-Thy-1*

Immunoprecipitated Thy-1 from thymocytes (lanes a and b) and lymph-node cells (lanes c and d) was either untreated (lanes a and d) or treated with neuraminidase (lanes b and c) before isoelectric focusing on a polyacrylamide slab gel containing Pharmalyte 3–10. For experimental details see the text.

not shown). The deglycosylated proteins from the two sources of cells appeared at the position of same apparent M_r, suggesting that no differences exist at the protein level. The conclusion from this experiment is that the differences between Thy-1 from thymocytes and lymph-node cells depend on the structures of the two 'complex-type' chains. Since the degree of sialylation was shown not to influence the migration on the gel, other structural differences must exist.

Differences in charge have been observed between Thy-1 antigens from mature and immature T-lymphocytes. Both types of cells produce Thy-1 molecules with various numbers of sialic acid residues, but the spectrum of bands of lymph-node-cell Thy-1 is shifted to the acidic side, as seen in analysis by isoelectric focusing (Hoessli *et al.*, 1980; Carlsson & Stigbrand, 1983*a*). Although the shift in isoelectric points has been proposed to be due to an increased sialylation, this has not been unequivocally shown. Fig. 2 shows an isoelectric-focusing experiment of intact and neuraminidase-treated Thy-1 antigens from thymocytes and lymph-node cells. The result clearly shows that both the charge heterogeneity and the acidic shift of lymph-node-cell Thy-1 are due to sialylation. As previously observed for thymocyte Thy-1 (Carlsson & Stigbrand, 1982, 1984), the digestion was not complete, which may be due to the presence of unusual sialic acid residues not susceptible to neuraminidase digestion. The results in Figs. 1 and 2 suggest that the branching of the carbohydrate chains of Thy-1 is decreased during maturation of the cells, and that coincidentally more sialic acid is built in.

Analysis of glycopeptides

In order to characterize further the differences in carbohydrate structure between Thy-1 from thymocytes and lymph-node cells, glycopeptides were prepared and analysed. Gahmberg & Andersson (1977) described a method to introduce tritium into sialic acid residues exposed at the cell surface. This method was used for labelling of thymocyte and lymph-node-cell glycoproteins in the present study. Fig. 3 shows a fluorograph of labelled glycoproteins separated by SDS/polyacrylamide-gel electrophoresis. On thymocytes three major components were labelled (Fig. 3, lane a). Besides Thy-1, two larger glycoproteins, identified as 'T-200' and 'leucocyte sialoglycoprotein' (for a review see Williams, 1982), were detected. The same components could also be identified with material from lymph-node cells, although Thy-1, probably because of the lower amounts present, was labelled to a much lesser extent (Fig. 3, lane c). Monospecific rabbit antibodies were used to immunoprecipitate Thy-1 from the cell lysates, and the isolated

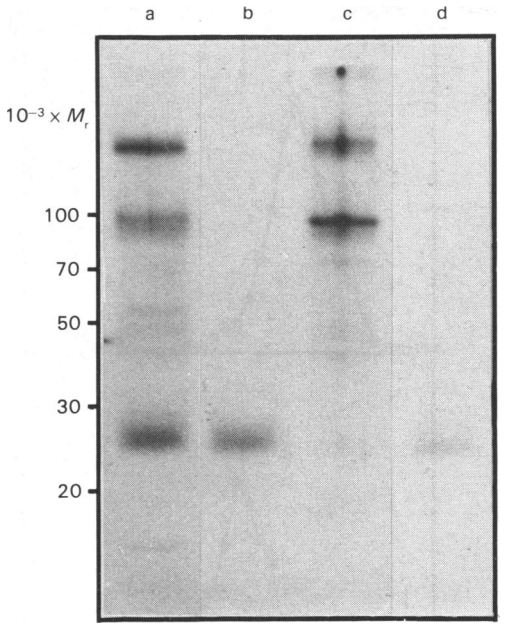

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of periodate/boro[^3H]hydride-labelled membrane glycoproteins*

Analysed material was from labelled thymocytes (lanes a and b) and lymph-node cells (lanes c and d). Lanes a and c show total cell lysates. Lanes b and d show immunoprecipitated Thy-1. For experimental details see the text.

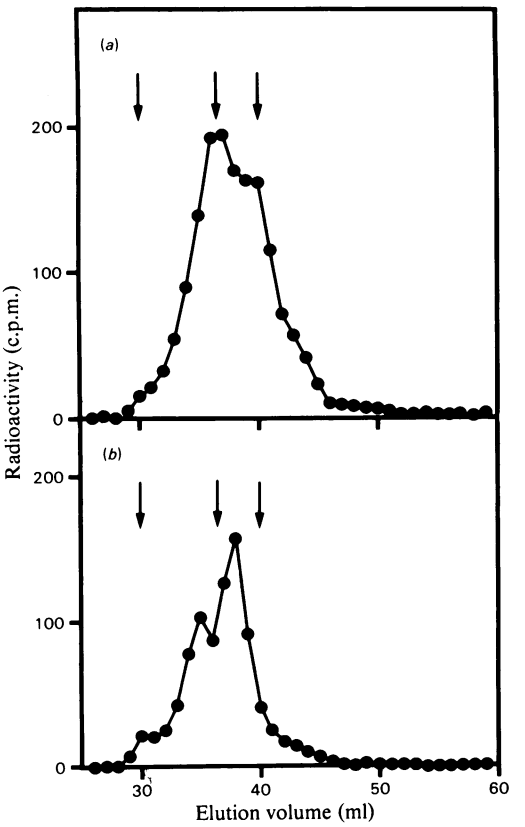

Fig. 4. *Gel filtration on Bio-Gel P-6 of Pronase-digested periodate/boro[^3H]hydride-labelled Thy-1*

(*a*) Thymocyte Thy-1; (*b*) lymph-node-cell Thy-1. The arrows show the elution of (from left to right): bovine serum albumin (V_0 marker) and [^{3}H]acetylated thymocyte Thy-1 glycopeptides I and IIA (Carlsson & Stigbrand, 1984). For experimental details see the text.

material is shown in Fig. 3 (lanes b and d). No bands other than Thy-1 were visible, and the immunoprecipitates could therefore be regarded as radioactive pure Thy-1. The proportion of Thy-1 immunoprecipitable from the lysates amounted to 8% and 3% of the total trichloroacetic acid-precipitable radioactivity from thymocytes and lymph-node cells respectively.

Immunoprecipitated Thy-1 was digested with Pronase and subjected to gel filtration on a Bio-Gel P-6 column, which was calibrated with glycopeptides obtained from purified thymocyte Thy-1 (Carlsson & Stigbrand, 1984). Figs. 4(*a*) and 4(*b*) show the elution of labelled glycopeptides from thymocytes and lymph-node cells. The elution profiles indicated the presence of triantennary and biantennary chains in both glycopeptide preparations, although in different proportions. These chains were separated by affinity chromatography on concanavalin A–Sepharose, which is known to separate 'complex-type' chains with differences in branching (Krusius *et al.*, 1976). The result is shown in Figs. 5(*a*) and 5(*b*). For thymocyte Thy-1 the proportions of unbound (representing triantennary chains, glycopeptide I) and sugar-eluted glycopeptides (representing biantennary chains, glycopeptide II) were 55% and 45% respectively. The extent of labelling of each type of chain probably depends on two factors, namely the total amount of each oligosaccharide and the number of sialic acid residues bound to the chain. In a previous study the amounts of triantennary and biantennary chains were determined to be 27% and 42% respectively of the total number of carbohydrate chains (Carlsson & Stigbrand, 1984). By assuming that all sialic acid residues were equally labelled, it can be calculated that the ratio of sialic acids of triantennary and biantennary chains is 2:1. For lymph-node-cell Thy-1, glycopeptides containing triantennary and biantennary chains amounted to 39% and 61% respectively. This means that the proportion of biantennary chains on Thy-1 is larger on lymph-node cells compared

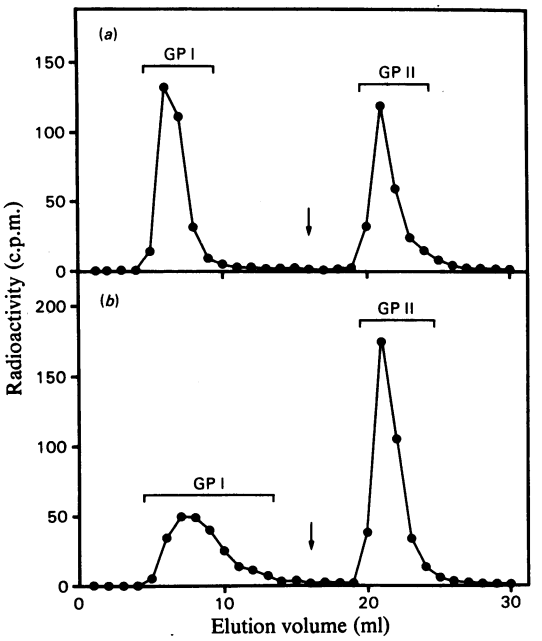

Fig. 5. *Separation of* [^3H]*glycopeptides on concanavalin A–Sepharose*

(*a*) Glycopeptides from thymocyte Thy-1; (*b*) glycopeptides from lymph-node-cell Thy-1. The arrows denote the beginning of elution with 25mM-methyl α-D-mannoside. Fractions were pooled as indicated by the bars. For experimental details see the text. Abbreviation: GP, glycopeptide.

with thymocytes, and/or that they are sialylated to a higher extent.

The size of the isolated glycopeptides was analysed by gel filtration (Fig. 6). Thymocyte glycopeptides I and II were eluted at the same positions as the marker glycopeptides for triantennary and biantennary chains respectively, confirming that the two types of chains were separated by concanavalin A–Sepharose chromatography. Lymph-node-cell Thy-1 glycopeptides I and II were both eluted earlier than the thymocyte Thy-1 counterparts, showing that the size of the respective glycan is slightly larger on lymph-node-cell Thy-1, which indicates that these chains contain more sialic acid. The conclusions from these experiments are therefore in agreement with the results shown in Figs. 1 and 2, that lymph-node-cell Thy-1 contains a higher proportion of biantennary chains than does thymocyte Thy-1. Furthermore, it is proposed that not only the biantennary chains but also the triantennary chains of lymph-node-cell Thy-1 contain more sialic acid than do the respective carbohydrate chains of thymocyte Thy-1.

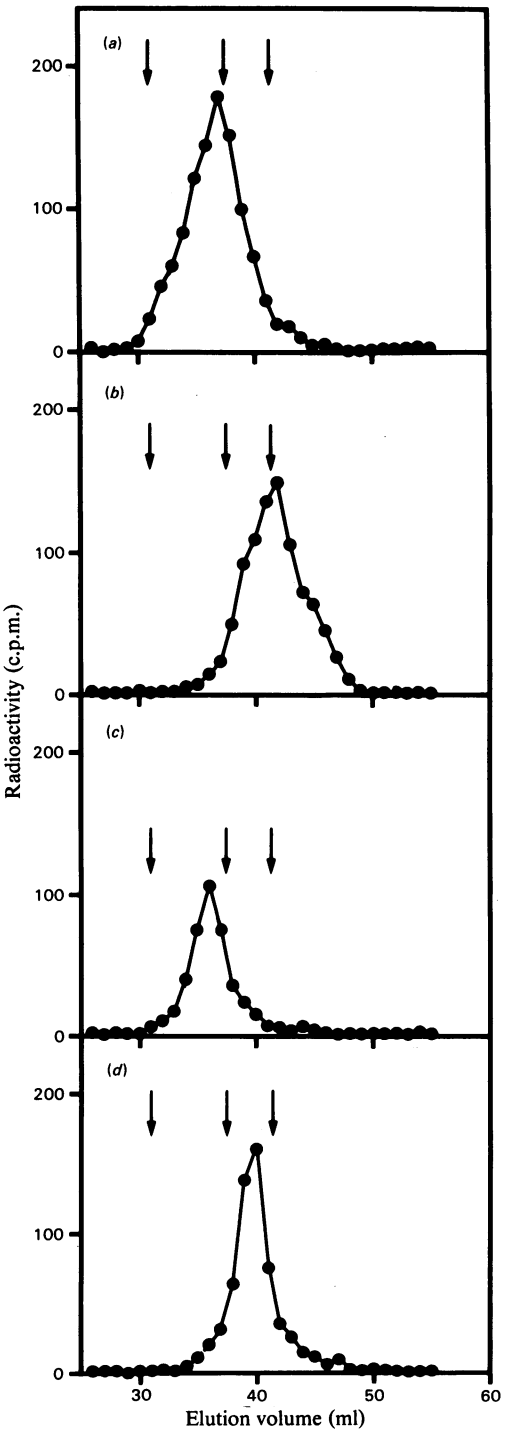

Fig. 6. *Analysis of concanavalin A-separated* [^3H]*glycopeptides by gel filtration on Bio-Gel P-6*

(*a*) Thymocyte Thy-1 glycopeptide I; (*b*) thymocyte Thy-1 glycopeptide II; (*c*) lymph-node-cell Thy-1 glycopeptide I; (*d*) lymph-node-cell Thy-1 glycopeptide II. The column was calibrated with the same compounds as indicated in the legend to Fig. 4.

Discussion

The structure of Thy-1 on mature and immature T-lymphocytes was studied with the aid of radioactive labelling of the cells, followed by immunoprecipitation and subsequent analysis by glycosidase digestions and glycopeptide isolation. The conclusions from the present study can be summarized as follows. Both thymocyte Thy-1 and lymph-node-cell Thy-1 are glycosylated by three *N*-linked carbohydrate chains. One of these chains on each molecule is of 'high-mannose' type, whereas the other two are 'complex-type' chains. The latter type of chains are both of triantennary and biantennary structure on Thy-1 of both cell types. The triantennary/biantennary ratio is higher on thymocyte Thy-1 than on lymph-node-cell Thy-1. This is reflected as a more pronounced heterogeneity visible on SDS/polyacrylamide-gel electrophoretograms. Both types of Thy-1 display variations in the sialic acid content. The two 'complex-type' chains of thymocyte Thy-1 together carry one to six sialic acid residues. Some molecules may also be devoid of sialic acid. The presence of six sialic acid residues may be accounted for by molecules that possess two triantennary chains where each branch is terminated by sialic acid. Lymph-node-cell Thy-1 carries on the average a larger number of sialic acid residues; the two 'complex-type' chains have together between two and seven residues of sialic acid. It follows that at least some of the biantennary chains, and perhaps also the triantennary chains, carry more than one sialic acid residue per branch.

An additional cause of the more pronounced heterogeneity of thymocyte Thy-1 compared with lymph-node-cell Thy-1 may be that the respective carbohydrate chain displays microheterogeneities other than those caused by sialic acid. For example, it has been shown that 50% of thymocyte Thy-1 glycopeptide II has a fucose residue linked to the innermost *N*-acetylglucosamine residue, whereas the other half has not (Carlsson & Stigbrand, 1984). This difference was too small to be detected by gel filtration, but variations at the terminals may give rise to detectable size heterogeneities. The broadness of the glycopeptide peaks obtained by gel filtration (Fig. 6) may, at least in part, be due to such heterogeneities. Lymph-node-cell Thy-1 glycopeptides gave sharper peaks, despite the fact that sialic acid heterogeneity exists. Moreover, lymph-node-cell Thy-1 appears as a sharper band on SDS/polyacrylamide-gel electrophoretograms (Fig. 1). Therefore three different effects may contribute to the heterogeneous appearance of thymocyte Thy-1: (1) variations in the branching pattern of the 'complex-type' chains (Carlsson & Stigbrand, 1984); (2) variations in the sialic acid content (Carlsson & Stigbrand, 1982); (3) other modifications at the branch terminals. On lymph-node-cell Thy-1 many of the triantennary chains have been replaced by biantennary chains, and Thy-1 molecules from these cells therefore lack the high-M_r forms. Sialic acid heterogeneity exists also on lymph-node-cell Thy-1, but other modifications may be less pronounced.

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