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Differences between the Carbohydrate Units of Cell-Surface Glycoproteins of Mouse B- and T-Lymphocytes

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Carbohydrate units of cell-surface glycoproteins of mouse B- and T-lymphocytes, labelled in their sialic acid residues by the periodate/NaB^3H_4 method and in their galactose residues by the galactose oxidase/NaB^3H_4 method after neuraminidase treatment, have been studied. Glycopeptides were prepared from the labelled cells by Pronase digestion and fractionated by concanavalin A affinity chromatography into two fractions (A and B). Alkali-labile oligosaccharides were isolated after mild NaOH/$NaBH_4$ treatment by gel filtration. The alkali-labile oligosaccharides were further analysed by t.l.c. To study the relative proportion of neutral mannose-rich carbohydrate units (fraction C) in lymphocyte glycoproteins, glycopeptides were also prepared from unlabelled cells and subjected to concanavalin A affinity chromatography after *N*-[^{3}H]acetylation of their peptide moiety. The major alkali-labile oligosaccharide component of both cell types was identified as galactosyl-(β1→3)-*N*-acetylgalactosaminitol. T-Lymphocytes were characterized by a high proportion of this oligosaccharide and a lower proportion of alkali-stable fraction A glycopeptides, whereas the opposite was observed for B-lymphocytes. The relative proportions of the concanavalin A-binding fractions B and C were similar in both cell types. The differences observed may correlate with the different surface properties of B- and T-lymphocytes.

The two major mouse lymphocyte subpopulations, the T- and B-cells, have been extensively studied. Numerous surface antigens and receptors have been defined (Möller, 1969). Most B-cells express receptors for complement component C3 and the Fc portion of immunoglobulin (Möller, 1969) and carry surface immunoglobulin. T-Cells, on the other hand, express the Thy-1,2 antigen (Möller, 1969). Their response to mitogen stimulation is also different. B-Cells are activated to divide *in vitro* by *Escherichia coli* lipopolysaccharide (Möller, 1972), whereas T-cells can be stimulated by various lectins like concanavalin A and phytohaemagglutinin (Möller, 1972). The surface-glycoprotein composition of these cells is also different (Trowbridge *et al.*, 1975; Gahmberg *et al.*, 1976). Although the surface glycoprotein patterns have been relatively well defined, there is very limited information available on the structure of their carbohydrate units.

In the present paper, we describe a comparative study of the structure of the carbohydrate units in glycoproteins of mouse B- and T-lymphocytes. The results show that the B- and T-cells differ in the relative amounts of different types of carbohydrate units present in their cell-surface glycoproteins.

Experimental

Preparation and fractionation of mouse B- and T-lymphocytes

Lymph nodes and spleens of mice were teased apart in cold RPMI 1640 culture medium. Clumps were removed by filtration through a loose cotton-wool plug. Erythrocytes were lysed with 0.83% NH_4Cl and phagocytic cells were removed with a magnet after incubation with carbonyl iron (Andersson *et al.*, 1973).

The purified lymphocytes were fractionated in a preparative free-flow-cell electrophoresis apparatus (type FF 4; Desaga, Heidelberg, Germany) at 6°C as described previously (Andersson *et al.*, 1973). After fractionation, more than 90% of the cells were viable as judged by the Trypan Blue-exclusion test. The purified B-cell fraction was contaminated by less than 2% of the Thy-1,2 antigen-carrying cells (T-cells), and correspondingly the T-cell fraction contained less than 2% surface-immunoglobulin-carrying cells (B-cells) (Andersson *et al.*, 1973).

Radioactive labelling of cells and glycopeptides

Cell-surface glycoproteins were specifically labelled

in their galactose residues after neuraminidase and galactose oxidase treatment with NaB^3H_4 as described previously (Gahmberg & Hakomori, 1973; Gahmberg *et al.*, 1976). The cells were also labelled in their surface sialic acid residues after mild periodate treatment followed by NaB^3H_4 (Gahmberg & Andersson, 1977). In separate experiments, glycopeptides prepared (see below) from unlabelled cells were *N*-[^{3}H]acetylated in their peptide moiety as described previously (Krusius, 1976).

Isolation of membrane proteins and preparation of glycopeptides

Lymphocytes (2×10^6 cells) were suspended in 10vol. of ice-cold 1% Triton X-100 in 0.15M-NaCl/0.01M-sodium phosphate, pH7.4, containing the proteinase inhibitor phenylmethanesulphonyl fluoride (2mM) and 1% ethanol and incubated on ice for 15min (Gahmberg & Andersson, 1978). Nuclei were removed by centrifugation at 1000*g* for 5min. The supernatant was freeze-dried. Lipids were extracted with 2×2ml of chloroform/methanol (2:1, v/v and 1:2, v/v respectively). The protein residue was digested with 2mg of Pronase (type VI proteinase; Sigma) in 2ml of 100mM-Tris/acetate buffer (pH7.8), containing 15mM-$CaCl_2$, at 45°C for 2 days (Arima & Spiro, 1972). The glycopeptides formed were purified by gel filtration on a column (1.3cm×25cm) of Sephadex G-25 (fine) and eluted with 10mM-pyridine/acetic acid buffer (pH5.0). Fractions of 2ml were collected and counted for radioactivity.

Affinity chromatography of glycopeptides on concanavalin A–Sepharose

Glycopeptides were fractionated on a column (1.0cm×1.0cm) of concanavalin A–Sepharose (Pharmacia) by stepwise elution with methyl α-D-glucoside as described previously (Krusius, 1976; Krusius *et al.*, 1976). Glycopeptides not bound to the lectin were eluted with the starting buffer, 5mM-sodium acetate (pH5.2), containing 0.1M-NaCl and $CaCl_2$, $MnCl_2$ and $MgCl_2$ (1mM each). Glycopeptides interacting weakly with concanavalin A were obtained by elution with 20mM-methyl α-D-glucoside and glycopeptides interacting strongly with the lectin by elution with 500mM-methyl α-D-glucoside, both in the starting buffer. Fractions of 1ml were collected and counted for radioactivity.

Separation of alkali-stable glycopeptides from alkali-labile oligosaccharides

Glycopeptides were *N*-acetylated in their peptide moiety to increase their alkaline sensitivity (Derevitskaya *et al.*, 1967) and treated with 2ml of 0.05M-NaOH in 1M-$NaBH_4$ for 16h at 45°C (Carlson, 1968). $NaBH_4$ was destroyed with glacial acetic acid. The released oligosaccharides were separated from the alkali-stable glycopeptides by gel filtration on a column (2cm×75cm) of Sephadex G-50 (fine), and eluted with 0.1M-pyridine/acetic acid buffer (pH5.0) (Krusius & Finne, 1978). Fractions of 4.6ml were collected and portions were counted for radioactivity.

Preparation of reference compounds for t.l.c.

Galactosyl-(β1→3)-*N*-acetylgalactosamine was prepared by partial acid hydrolysis from brain gangliosides (Finne *et al.*, 1977). The oligosaccharides were reduced by incubation in 25mM-NaB^3H_4 (sp. radioactivity 230mCi/mmol; New England Nuclear) at room temperature (20°C) for 3h. NaB^3H_4 was destroyed with glacial acetic acid, the reduced oligosaccharides were desalted by passing through columns (0.8cm×4cm) of Dowex-50 and Dowex-1 and taken to dryness. Borate was removed by repeated evaporations with methanol/acetic acid (1000:1, v/v). Galactosyl-(β1→3)-*N*-acetylgalactosaminitol was finally purified by preparative t.l.c. on silica gel G plates developed with chloroform/methanol/2.5M-NH_4OH (60:35:8, by vol.). The structure of the disaccharide was confirmed by g.l.c. and mass spectrometry (Finne *et al.*, 1977). Galactitol, lactitol, *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol were prepared similarly by NaB^3H_4 reduction from the parent reducing saccharides.

T.l.c.

T.l.c. was performed on DC-Alufolien Kieselgel 60 plates (Merck). The solvents were: A, chloroform/methanol/2.5M-NH_4OH (60:35:8, by vol.); B, n-propanol/water (7:3, v/v); and C, n-propanol/ethyl acetate/water (5:1:1, by vol.). After development the plates were cut into segments of 2.5mm and counted for radioactivity in 10ml of Bray's scintillation solution.

Results

Cell-surface glycoproteins of mouse B- and T-lymphocytes were labelled with NaB^3H_4 in their sialic acid or galactose residues after treatment with periodate or neuraminidase and galactose oxidase respectively. Glycopeptides were prepared by Pronase digestion from the Triton X-100-solubilized membrane proteins and purified by gel filtration on Sephadex G-25. The purified glycopeptides were fractionated by concanavalin A–Sepharose chromatography under standardized conditions that have previously been shown to produce fractions containing structurally distinct glycopeptides (Krusius & Finne, 1978). The glycopeptides labelled either in their

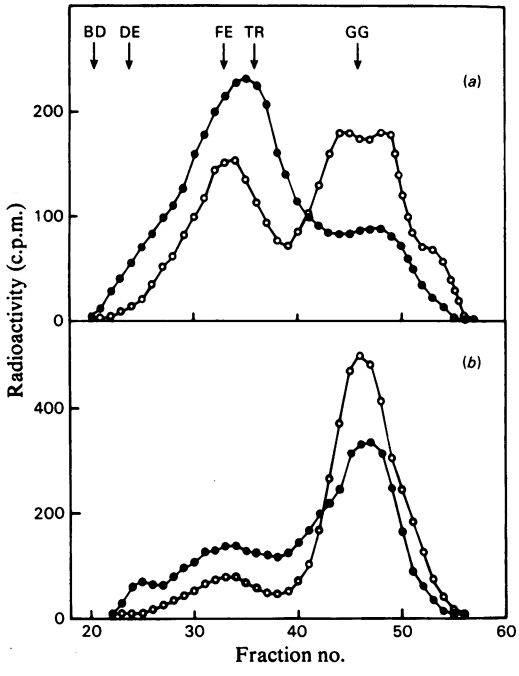

Fig. 1. *Gel filtration of cell-surface-derived glycopeptides of B- and T-cells labelled after neuraminidase and galactose oxidase treatment with NaB^3H_4*

The glycopeptides that were not bound to concanavalin A were fractionated on a column (2cm×75cm) of Sephadex G-50, before (●) and after (○) mild treatment with NaOH/$NaBH_4$. The column was eluted with 0.1M-pyridine/acetic acid buffer (pH5.0). Fractions of 4.6ml were collected and portions of 0.9ml were counted for radioactivity. For comparison the elution volumes of Blue Dextran (BD), Dextran T 10 (DE, M_r 9400), fetuin glycopeptide [FE, M_r 3400 (Bayard, 1974)], transferrin glycopeptide [TR, M_r 2400 (Spik *et al.*, 1975)] and galactosyl-(β1→3)-*N*-acetylgalactosaminitol (GG, M_r 385) are shown. (*a*) shows glycopeptides from B-lymphocytes and (*b*) shows glycopeptides from T-lymphocytes.

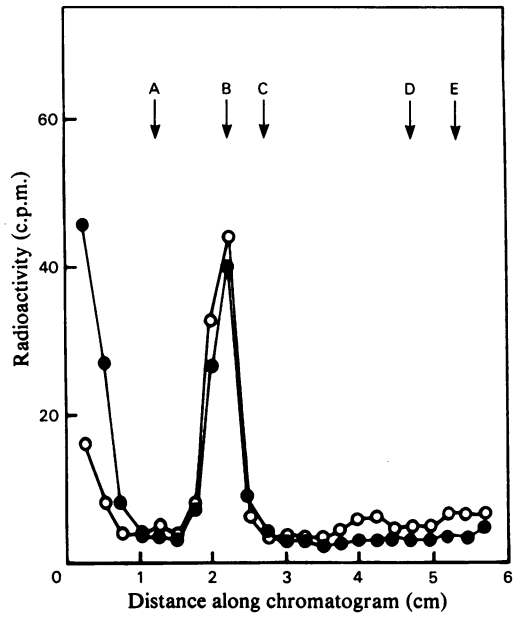

Fig. 2. *T.l.c. of alkali-labile oligosaccharides labelled in their galactose residues*

The oligosaccharides were isolated after mild treatment with NaOH/$NaBH_4$ by gel filtration as shown in Fig. 1. The chromatogram was developed with solvent A, cut into segments of 2.5mm and counted for radioactivity. For comparison the positions of lactitol (A), galactosyl-(β1→3)-*N*-acetylgalactosaminitol (B), galactitol (C), *N*-acetylglucosaminitol (D) and *N*-acetylgalactosaminitol (E) are shown. Symbols: ●, B-lymphocytes; ○, T-lymphocytes.

galactose or sialic acid residues produced two fractions. The majority of the glycopeptides was not bound to concanavalin A. A proportion (20%) of the galactose label from B-cells and 9% from T-cells was eluted with 20mM-methyl α-D-glucoside (fraction B). No radioactivity was eluted with 500mM-methyl α-D-glucoside, which displaces neutral fraction C glycopeptides (Krusius, 1976; Krusius & Finne, 1978). Of the radioactivity 85–90% applied to the column was recovered. Affinity chromatography of B- and T-cells glycopeptides labelled in their sialic acid residues produced similar results.

The galactose-labelled glycopeptides not bound to concanavalin A were subjected to gel filtration on Sephadex G-50 before and after treatment with NaOH/$NaBH_4$ (Fig. 1). Two peaks were obtained. The first peak (fraction A) eluted between the positions of Dextran T 10 and transferrin glycopeptide, which correspond to mol.wts. of 9400 and 2400 (Spik *et al.*, 1975), was probably composed of *N*-glycosidic glycopeptides. The second peak represented alkali-labile oligosaccharides. The elution volume of this peak corresponded to that of galactosyl-(β1→3)-*N*-acetylgalactosaminitol. The relative proportions of the two peaks were about the same in B-cells, whereas T-cells contained considerably more alkali-labile oligosaccharides.

Glycopeptides labelled in their sialic acid residues and not bound to concanavalin A were also chromatographed before and after treatment with NaOH/$NaBH_4$ on Sephadex G-50. Similar elution profiles were obtained as for galactose-labelled glycopeptides from B- and T-cells. This indicated that carbohydrate

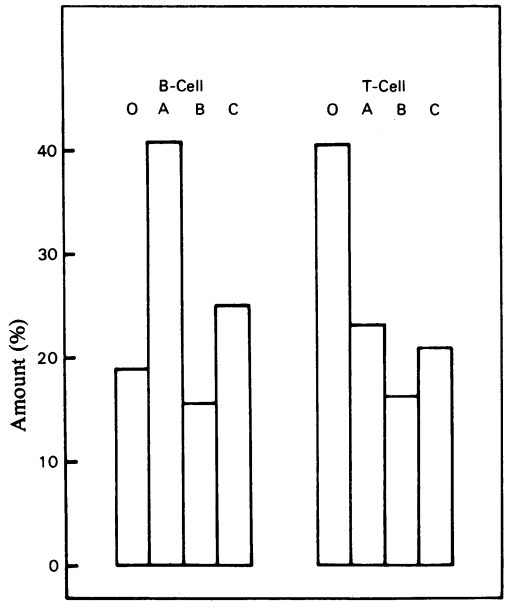

Fig. 3. *Relative proportions of galactosyl-(β1→3)-N-acetylgalactosaminitol* (○) *and alkali-stable glycopeptide fractions (A, B and C) prepared from B- and T-lymphocytes*
The alkali-labile oligosaccharide and fraction A and B glycopeptides were isolated from cells labelled by the galactose oxidase/NaB^3H_4 method. Fraction C glycopeptides were prepared from unlabelled cells and their proportion was determined after *N*-[^{3}H]-acetylation and concanavalin A chromatography.

units of B- and T-cells contained an approximately similar proportion of sialic acid residues, compared with galactose residues.

The alkali-labile oligosaccharides from B- and T-lymphocytes were further analysed by t.l.c. (Fig. 2). Only one major radioactive oligosaccharide was detected from the galactose-labelled B- and T-cells. The peak had the same R_F value as galactosyl-(β1→3)-*N*-acetylgalactosaminitol in three different solvent systems. The radioactivity seen at the origin (Fig. 2) is probably due to the presence of alkali-stable glycopeptides. About 60% of the radioactivity present in the oligosaccharide peak from Sephadex G-50 chromatography migrated near the solvent front, faster than reference mono- and di-saccharides (not seen in Fig. 2), suggesting that the radioactivity was not bound to carbohydrate but to amino acids and other low-molecular-weight substances.

Sugars labelled by the galactose oxidase/NaB^3H_4 method were identified by t.l.c. after hydrolysis of glycopeptides (pooled fractions A and B) and isolated galactosyl-(β1→3)-*N*-acetylgalactosaminitol in 2M-HCl at 100°C for 2h, re-*N*-acetylation and reduction. Only one radioactive component was observed, which co-migrated in three solvents with galactitol.

To study the presence of neutral-type (fraction C) carbohydrate units in glycoproteins of B- and T-cells, glycopeptides were prepared from unlabelled cells by Pronase digestion. The purified glycopeptides were *N*-[^{3}H]acetylated in their peptide moiety and chromatographed on concanavalin A–Sepharose. The ratio of radioactivity eluted with 500mM-methyl α-D-glucoside (fraction C) to that obtained with 20mM-methyl α-D-glucoside (fraction B) was 1.6:1.0 in B-cells and 1.3:1.0 in T-cells. Taking into account the relative amounts of different concanavalin A fractions and the radioactivity present in galactosyl-(β1→3)-*N*-acetylgalactosaminitol purified by t.l.c., the relative proportions of the different fractions can be summarized (Fig. 3). The major difference in B- and T-lymphocyte membrane glycoproteins is the relative proportion of alkali-labile oligosaccharides and fraction A glycopeptides. Fraction A glycopeptides, which probably represent acidic-type *N*-glycosidic glycopeptides with three or more peripheral branches (Krusius *et al.*, 1976; Krusius & Finne, 1978), are enriched in B-cells, whereas alkali-labile oligosaccharides predominate in cell-surface glycoproteins of the T-cells. The relative amounts of the fraction B glycopeptides, which are supposed to have two peripheral branches (Krusius *et al.*, 1976; Krusius & Finne, 1978), and of the neutral-fraction C glycopeptides are similar in both cell types.

Discussion

Owing to difficulties in the isolation and fractionation of lymphocytes in large amounts, information on the nature of the carbohydrate units of their cell-surface glycoproteins is limited. The carbohydrate composition of plasma membrane preparations obtained from pig lymphocytes has been determined (Snary *et al.*, 1976). Newman *et al.* (1976) identified the disaccharide galactosyl-(β1→3)-*N*-acetylgalactosaminitol after desialylation and alkaline borohydride treatment from lymphocytes of pig peripheral blood. Saito *et al.* (1977) have isolated and partially characterized one *N*-glycosidic glycopeptide and two *O*-glycosidic glycopeptides from mitogen-stimulated human-peripheral-blood lymphocytes. Recently Kornfeld (1978) characterized three acidic-type *N*-glycosidic glycopeptides from calf thymocyte plasma membranes. These glycopeptides resemble the acidic-type *N*-glycosidically linked carbohydrate units present in soluble glycoproteins (Montreuil, 1975).

No comparative studies on the structure of the carbohydrate units of glycoproteins of different lymphocyte subpopulations have been reported. However, it may be expected that structural differences occur, since lectins selectively stimulate

different lymphocytes to divide (Möller, 1972). Furthermore, mouse lymphocytes show specific and characteristic surface-glycoprotein patterns (Trowbridge *et al.*, 1975; Gahmberg *et al.*, 1976). Therefore the present study was undertaken to compare the carbohydrate units of cell-surface glycoproteins of B- and T-lymphocytes.

Glycopeptides prepared by Pronase digestion from the labelled plasma membrane proteins of B- and T-cells were subjected to a recently developed fractionation procedure by which it has been possible to isolate structurally distinct glycopeptide fractions from a variety of sources (Krusius & Finne, 1978).

Glycopeptides labelled in either their sialic acid or galactose residues were observed in three different fractions (O, A and B). No radioactivity was found in fraction C, which represents neutral mannose-rich glycopeptides (Krusius, 1976; Krusius & Finne, 1978). However, when glycopeptides were prepared from unlabelled glycoproteins and were *N*-[^{3}H]-acetylated in their peptide moiety before concanavalin A affinity chromatography the latter fraction also became labelled. Thus it became possible to determine the proportion of these glycopeptides as well.

Fraction A glycopeptides, which probably represented acidic *N*-glycosidic glycopeptides with three or more neuraminylgalactosyl-*N*-acetylglucosamine branches (Krusius *et al.*, 1976; Krusius & Finne, 1978), accounted for 44% and *O*-glycosidic oligosaccharides for 22% of the galactose label in B-cells, whereas in T-cells fraction A glycopeptides corresponded to 26% and *O*-glycosidic oligosaccharides to up to 44% of the label. The relative amount of fraction B glycopeptides that represented acidic-type *N*-glycosidic glycopeptides with two peripheral branches (Krusius *et al.*, 1976; Krusius & Finne, 1978) and the proportion of neutral-type glycopeptides (fraction C) were similar in B- and T-lymphocytes.

Previous studies show that most of the surface label after neuraminidase and galactose oxidase treatment in T-lymphocytes was in two major glycoproteins with apparent mol.wts. of 180000 and 125000. B-Cells contained only one highly labelled surface glycoprotein with an apparent mol.wt. of 210000 (Gahmberg *et al.*, 1976). Whether the two main glycoproteins in T-cells contain similar or different types of carbohydrate chains is not known.

On t.l.c. only one radioactive alkali-labile oligosaccharide was found both from B- and T-cells. The oligosaccharide was identified as galactosyl-(β1→3)-*N*-acetylgalactosaminitol from its relative mobilities on t.l.c. in three different solvent systems. This disaccharide and its sialosylated derivatives have also been found to be the major components of the alkali-labile oligosaccharide fraction isolated from a variety of different sources (Finne & Krusius, 1976; Newman *et al.*, 1976; Saito *et al.*, 1977; Krusius & Finne, 1978). Owing to scarcity of material the alkali-labile oligosaccharides labelled in their sialic acid residues were not analysed on t.l.c. However, two findings suggest that the galactosyl-(β1→3)-*N*-acetylgalactosaminitol present in both B- and T-cells occurred as sialosylated derivatives of the disaccharide. First, the alkali-labile oligosaccharides labelled by the sodium periodate/NaB^3H_4 method were eluted earlier than galactosyl-(β1→3)-*N*-acetylgalactosaminitol. This elution volume corresponded approximately to the elution volumes of mono- and di-sialosyl derivatives of the core disaccharide (Krusius & Finne, 1978). Secondly, we have previously found that almost no label is incorporated into the glycoproteins after treatment with galactose oxidase without neuraminidase (Gahmberg *et al.*, 1976).

The present results show that the lack of stimulation of B-lymphocytes by concanavalin A is not due to absence of concanavalin A-binding carbohydrate units from B-cells. Therefore other reasons like binding to specific surface glycoproteins involved in growth stimulation or differences in their ability to redistribute on the cell surface must be taken into consideration.

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