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EVIDENCE FOR THE OCCURRENCE OF O-GLYCOSIDICALLY LINKED OLIGOSACCHARIDES OF POLY-N-ACETYLLACTOSAMINE TYPE ON THE HUMAN LEUCOCYTE COMMON ANTIGEN

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Summary High molecular weight glycoproteins of human B and T lymphocytes known as leucocyte common antigen or T200 have been shown to carry O- and N-glycosidically linked, sialylated, carbohydrate chains. The O-linked chains are polydisperse and those of B rather than T cell type are highly susceptible to degradation by endo-β-galactosidase. These differences among lymphocytes that are functionally distinct raise the possibility that the oligosaccharides may contribute to the functions of these differentiation molecules as well as to their electrophoretic diversity.

Ubiquitous among leucocytes is a family of high molecular weight glycoproteins (1-4) known as leucocyte common antigen (LC) or T200. These glycoproteins resolve into multiple bands upon SDS-polyacrylamide gel electrophoresis which change following stimulation of lymphocytes and vary in lymphocyte clones that are functionally distinct (5-9). The LC glycoproteins of B lymphocytes have a higher average mol. wt. (220K) compared with those of T cells (200K) (ref. 10,11). The B and T forms of these glycoproteins also differ in their antigenicities and reactivities with plant lectins (6,10-12). The biochemical basis of these antigenic differences and the differing banding patterns is not yet known. In a recent study (13) we showed that high molecular weight glycoproteins (>200K) of B rather than T lymphocytes reacted strongly with anti-I and -i antibodies which recognize (14) oligosaccharide sequences of poly-N-acetyllactosamine type. However, these glycoproteins were not specifically identified as LC molecules. In the present studies we have confirmed and extended these observations using specifically isolated (11) LC molecules of B and T cell

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types. We also have preliminary evidence that both the T and B lymphocyte forms of LC glycoproteins have two categories of carbohydrate side chains: an alkali stable category corresponding to the previously noted (15) N-glycosidically linked chains and a hitherto undetected category which is alkali labile (O-glycosidically linked) (16).

MATERIALS AND METHODS

Isolation of LC glycoproteins and affinity chromatography on an anti-i antibody adsorbent. Human lymph node lymphocytes were surface labelled by the $NaIO_4/NaB[^3H]_4$ method which labels sialic acid residues (17). LC glycoproteins of B and T cell types (average mol. wts. 220K and 200K, respectively) were isolated by sequential affinity chromatography on F8-11-13 followed by F10-89-4 antibody adsorbents (11,18). The former hybridoma antibody reacts with a predominantly B lymphocyte specific determinant on the human LC molecule and the latter reacts with all forms of the human LC molecule (11). Evidence was obtained that the labelled monosaccharide in the eluted glycoproteins was sialic acid as follows: the main radioactive counts released after treatment of peak I glycopeptides with alkali and peak II glycopeptides with acid corresponded to sialic acid (see legend to Fig. 2).

The radioactive LC glycoproteins (7000 cpm) of B and T cell types were passed at 4°C over an anti-i(Den)-Sepharose 4B adsorbent column or a control human IgM-Sepharose 4B column consisting of 4 mg IgM on 2ml of Sepharose 4B. After washing at 4°C the radioactivity bound was eluted at 37°C as described previously (19).

Pronase digestion of LC glycoproteins and gel filtration. One hundred µg of pronase (Calbiochem B grade) were added to LC preparations (approx 1×10^5 cpm) of B or T cell types in 2 ml of 0.15M Tris/acetate buffer, pH 7.7, containing 2 mM $CaCl_2$, 0.5% SDS and 2 mg human Cohn fraction II (Miles, Slough, UK) and incubated at 37°C. Further pronase (100 µg) was added after 24 h and 48 h. Digestion was terminated after 72 h by heating at 100°C for 5 mins. Digests of LC glycoproteins were applied to a Sephadex G50 column (1.5 x 90 cm) equilibrated in 0.15M NaCl, 0.02% NaN_3; flow rate was 20 ml/h. Fractions, 2 ml, were pooled to give peak I and II glycopeptides as indicated by the solid bars (Fig. 1).

Treatment of pronase glycopeptides with endo-ß-galactosidase, alkali or alkaline borohydride. Samples of LC glycopeptides (approx. 3000 cpm) in 1 ml 50 mM sodium acetate buffer, pH 5.8, containing 0.1 mg/ml bovine serum albumin were digested with endo-ß-galactosidase (20) from B. fragilis (250 mU) at 37°C for 24 h. Our recent studies, to be described in detail elsewhere, have shown that this enzyme has similar specificity to the endo-ß-galactosidase of E. freundii (21). Samples (approx. 3000 cpm) for alkali treatment were incubated in 0.1N NaOH at 37°C for 48 h and neutralized with HCl prior to gel filtration as described above. Samples (2000 - 3000 cpm) treated with alkaline borohydride were incubated in 0.1N NaOH containing 1M $NaBH_4$ at 37°C for 48 h. The $NaBH_4$ was destroyed by the addition of acetic acid and borate was removed by repeated addition and evaporation of 1% acetic acid in methanol.

Identification of reduced tritium labelled hexosamines obtained from alkaline borohydride treated peak I glycopeptides. Samples of peak I glycopeptides (approx. 1000 cpm of sialic acid label) were treated with 0.05N NaOH containing 1M $NaBH_4$ and 5 mCi $NaB[^3H]_4$ at 50°C for 18 h, neutralized with acetic acid, evaporated several times with methanol containing 1% glacial acetic acid and hydrolysed in 4N HCl at 100°C for 6 h. The hexosamine and hexosaminitol fractions were purified by cation exchange chromatography, re-N-acetylation and desalting as described (22). The samples were each divided for duplicate analysis by tlc with standard hexosaminitols (prepared as above) using high performance plates (HP-KF Whatman, Uniscience, Cambridge, UK) impregnated with borate and run in solvent system (22) butyl acetate/isopropanol/pyridine/water, 7:3:2:2 (v/v), until the solvent front reached 18 cms from the origin. Radioactivity in 2 mm strips scraped from the plates was measured by liquid scintillation.

RESULTS AND DISCUSSION

LC glycoproteins of both B and T cell types were tested for binding to an anti-i adsorbent column (Fig. 1A). Glycoproteins of both types bound to the

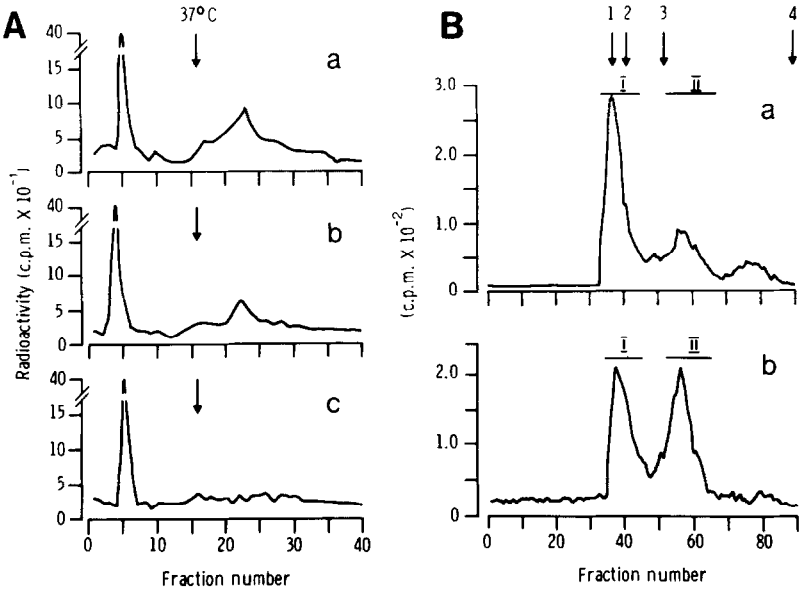


Fig. 1 A. The binding of [^{3}H]-labelled LC glycoproteins of B and T cell types to anti-i adsorbent (a and b, respectively) or to a control adsorbent (c, LC glycoproteins of T cell type).
B. Gel filtration on Sephadex G50 of [^{3}H]-labelled pronase glycopeptides of LC molecules of B and T cell type (a and b, respectively). Molecular weight markers: 1, Blue Dextran 2000 (Pharmacia), excluded volume; 2, Dextran T10, average mol. wt. 10,000; 3, fetuin pronase glycopeptides, average mol. wt. 3000; 4, NaB[^{3}H]$_4$.

adsorbent column and could be specifically eluted at 37°C. A higher proportion of the B type molecules (13%) bound than those of T type (6%). This and our previous study (13), using neuraminidase treated lymphocytes show that the strong expression of i antigen on LC molecules of B type is manifest both before and after removal of sialic acid residues. Thus the low expression of this antigen on LC molecules of T type is unlikely to be due to a masking effect (14) of sialic acid residues.

Pronase digestion of LC molecules of both B and T cell types revealed two families of sialoglycopeptides (peaks I and II, Fig. 1B). Peak I, which was largely excluded from a Sephadex G50 column, represents glycopeptides with apparent molecular weights >10,000; the included peak II eluted behind pronase glycopeptides of fetuin and corresponds to glycopeptides with molecular weights of approximately 2000. The relative amounts of radioactivity in the two peaks were different in LC molecules of B and T cell types. Peak I was larger than peak II in the glycoproteins of B type but the two peaks were of approximately equal size in the T type glycoproteins. Since sialic acid is the only labelled

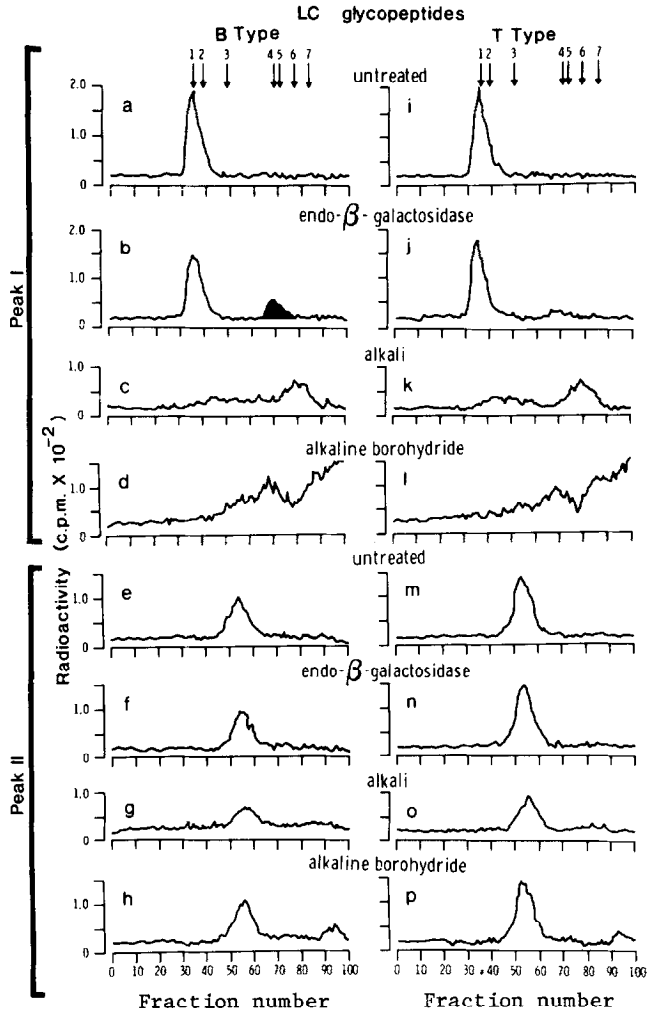


Fig. 2 Chromatography on Sephadex G50 of peaks I and II pronase glycopeptides of LC molecules of B and T cell type before and after treatment with endo-β-galactosidase, alkali or alkaline borohydride. Arrows indicate positions of molecular weight markers; 1, Blue Dextran 2000, excluded volume; 2, Dextran T10, mol. wt. 10,000; 3, fetuin pronase glycopeptides mol. wt. 3000; 4, tetrasaccharide SAα2→3Galβ1→4GlcNAcβ1→3Gal released from sialylparagloboside (21) by endo-β-galactosidase (B. fragilis) digestion; 5, N-acetylneuraminlactose: SA2→3(6)Galβ1→4Glc (Sigma); 6, N-acetylneuraminic acid: this was the elution position of radioactivity released after acid treatment (0.15N HCl, 80°C, 30 min) of peak II glycopeptides of T cell type; 7, $NaB[^3H]_4$.

monosaccharide in these studies (see Materials and Methods and Fig. 2), it is not possible to say whether the peak heights reflect differences in the number of oligosaccharide chains or the degree of their sialylation.

The peak I glycopeptides of B and T types differed in their susceptibility to digestion by endo-β-galactosidase of B. fragilis. Approximately 20% of the radioactivity of peak I glycopeptides of B type was released (Fig. 2b shaded area) yielding a peak which co-chromatographed with the tetrasaccharide

SAα2→3Galβ1→4GlcNAcβ1→3Gal. Treatment of peak I glycopeptides of T cell type with endo-β-galactosidase released only 7% of the radioactivity eluting in the tetrasaccharide region (Fig. 2j). In contrast to peak I glycopeptides, the elution profiles of the peak II glycopeptides of both B and T cell types were unaffected by the endo-β-galactosidase digestion (Fig. 2f and n).

Treatment of peak I glycopeptides of B and T cell type with 0.1N NaOH, which is known (16) to release oligosaccharides joined by O-glycosidic linkage (β-elimination) and degrade them (peeling reaction) into smaller oligosaccharides of varying size, resulted in complete release of the oligosaccharides (Fig. 2c and k). The majority of the radioactivity released eluted in fractions 72-86 corresponding to free sialic acid. In contrast, the oligosaccharides in peak II behaved like N-linked chains which were unaffected by the alkaline treatment (Fig. 2g and o). In the presence of 0.1N NaOH and 1M $NaBH_4$ both N- and O-glycosidically linked chains can be released from protein and further degradation of the chains is minimized (22,23). Under these conditions the peak II profiles showed no change (Fig. 2h and p) while the peak I glycopeptides of B and T types yielded a spectrum of radioactive counts in the apparent mol. wt. range 300 to 2600, corresponding to sialylated oligosaccharides containing up to 12 monosaccharides (Fig. 2d and l). The majority of the radioactivity associated with oligosaccharides eluted with a peak corresponding to sialylated tetrasaccharide. These results suggest strongly that the peak I glycopeptides are pronase resistant because they contain clusters of O-glycosidically linked oligosaccharides. The degradation procedure also released variable proportions of radioactivity which chromatographed as tritiated salt and requires investigation.

The main reduced monosaccharide obtained after alkaline borohydride degradation and hydrolysis of peak I glycopeptides co-chromatographed with the standard N-acetylgalactosaminitol (Fig. 3). Some N-acetylglucosaminitol may also be present. As core N-acetylgalactosamine has in the past been found linked to the oxygen of serine or threonine amino acids this finding is further evidence for the O-glycosidic nature of carbohydrate chains in peak I.

It is interesting that, until now, the alkali labile carbohydrate chains of LC glycoproteins have gone undetected. However, the total oligosaccharide or

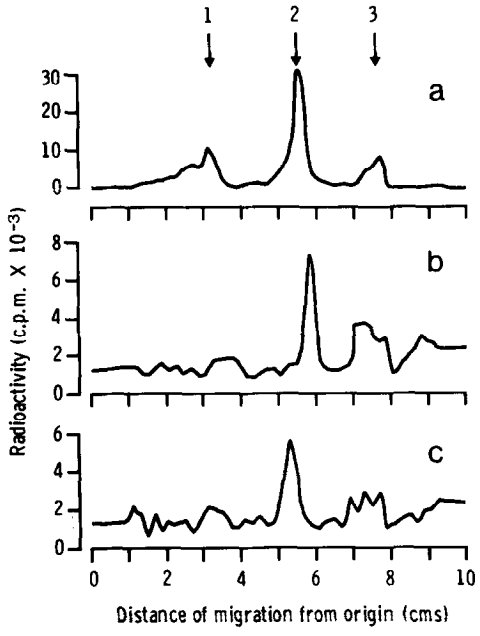
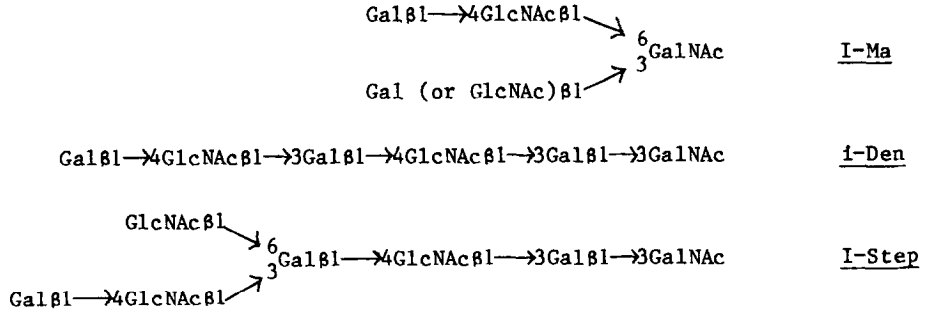


Fig. 3 Thin layer chromatography of reduced ^{3}H-labelled hexosamines obtained from alkaline borohydride treated peak I pronase glycopeptides of B and T cell types. (a) standard reduced hexosamines; arrows indicate position of N-acetylglucosaminitol (1); N-acetylgalactosaminitol (2); unidentified peak arising from N-acetylgalactosaminitol (3). (b) Peak I glycopeptides of B cell type. (c) Peak I glycopeptides of T cell type.

glycopeptide profiles reported in previous studies (15,24) seem compatible with a mixture of the alkali-stable and alkali-labile oligosaccharides detected here.

From knowledge of the carbohydrate sequences (14) recognized by anti-i antibody (Den) used in this study and two anti-I antibodies (Ma and Step) used in our previous study (13) we can propose the minimum structures on peak I glycopeptides that would express the three types of antigenic determinant. The inference from this study is that these structures are expressed on O-glycosidically linked oligosaccharides. These would consist of a tetra-, hexa- and heptasaccharide as follows:



Susceptibility to endo-β-galactosidase is likewise a property associated with tetrasaccharides or longer oligosaccharides of the poly-N-acetyllactosamine series (21). At present, we do not know the structural differences between the O-linked oligosaccharides of the T and B forms of LC glycoproteins that account for the relative lack of Ii activities on the T form and also the relative resistance to endo-β-galactosidase. These differences are not necessarily due to shorter chain lengths in the LC glycoproteins of T cell types (Fig. 2d,1) but they may be due to structural differences in the 'backbone' regions (25) of these oligosaccharides or the masking effect (14) of certain monosaccharides, such as fucose, in their 'peripheral' regions.

The peak I glycopeptides resemble high molecular weight gastric (25) and ovarian cyst (26) glycoproteins (mucins) in having alkali labile oligosaccharide chains possibly up to twelve sugars long. This is in contrast to the O-glycosidically linked chains thus far described on membrane glycoproteins, the majority of which range from mono- to hexasaccharides (27-30). However, hepta- to decasaccharides, containing poly-N-acetyllactosamine sequences have been reported recently (31) on O-linked chains of glycophorin of bovine erythrocytes.

Multiple, sialylated, O-glycosidically linked oligosaccharide chains of varying length could substantially affect the apparent molecular weight of LC molecules and it is possible that these oligosaccharides are responsible for the apparent heterogeneity of LC glycoproteins seen in SDS-polyacrylamide gels. Furthermore, the differences in the properties of the carbohydrate chains of LC glycoproteins of B and T cell type, e.g. their reactions with anti-I and -i antibodies, lectins and the susceptibility of their sialylated chains to endo-β-galactosidase, suggest that the carbohydrate moieties may contribute to the functions of these glycoproteins.

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