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Evidence from Lectin-binding Studies for Abnormal Glycosylation of β-Hexosaminidase in the Leukaemic Cell-Line CCRF/CEM

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The lectin affinities of β-*N*-acetyl-D-hexosaminidase (E.C.3.2.1.52) from an acute lymphoblastic leukaemic cell-line (CCRF/CEM), a non-malignant lymphoblastic cell-line (SM1) and normal human fibroblasts were studied for both mature and precursor forms of the enzyme. Four immobilised lectins, concanavalin A-Sepharose, wheat germ agglutinin-Agarose, *Ricinus communis* agglutinin I-Agarose, *Phaseolus vulgaris* erythroagglutinin-Agarose and a column of serotonin-Sepharose were used. The activities of β-hexosaminidase from fibroblasts and SM1 cells generally behaved similarly while the CCRF/CEM enzyme exhibited different binding patterns. Differences were also noted between precursor and mature enzyme from each cell type consistent with changes in glycosylation between the precursor form and the mature form appearing in the lysosome. These results suggest that changes in the glycosylation of β-hexosaminidase, and possibly other lysosomal enzymes, may be associated with malignancy.

The presence of unusual lysosomal enzymes in lymphoblasts from most patients with non-T, non-B cell acute lymphoblastic leukaemia (non-T, non-B ALL) is well recognised [1-6] and lysosomal enzyme analysis has become an increasingly useful supplement to cytochemical and immunological techniques for the diagnosis of both lymphoid and non-lymphoid leukaemia [5, 6]. Of the lysosomal enzymes studied, β-hexosaminidase has proved the most useful as a phenotype marker, and the intermediate form, β-hexosaminidase I, observed on isoelectric focusing [4, 6] or DEAE-cellulose chromatography [1, 7], is characteristic of the non-T, non-B ALL phenotype.

Abbreviations. Con A; concanavalin A-Sepharose: RCA-I; *Ricinus communis* agglutinin I-Agarose: WGA; wheat germ agglutinin-Agarose: PHA-E; *Phaseolus vulgaris* erythroagglutinin-Agarose: SER; serotonin-Sepharose: non-T, non-B ALL; non-T, non-B cell acute lyphoblastic leukaemia: 4-MU-GLcNAc; 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside.

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Little is understood of the biochemistry of atypical β-hexosaminidase isoenzymes, but because several lysosomal enzymes are similarly affected [4], and almost all are glycoproteins [8], a general aberration in glycosylation is the most probable cause. We have previously demonstrated that the cell-line CCRF/CEM derived from a patient with non-T, non-B ALL, expresses the abnormal lysosomal enzyme profiles characteristic of that phenotype [9], and therefore provides a useful source of enzyme for investigation. In this study we used the discriminatory sugar-binding capacity of lectins to identify differences in glycosylation between β-hexosaminidase from malignant and non-malignant sources. We also examined precursor forms of β-hexosaminidase to ascertain whether differences in glycosylation were evident at an earlier stage of biosynthesis, prior to recognition and targeting to the lysosome.

Materials and Methods

The following materials were obtained from the suppliers indicated. *Phaseolus vulgaris* erythroagglutinin-Agarose (PHA-E), wheat germ agglutinin-Agarose (WGA) and *Ricinus communis* agglutinin I-Agarose (RCA-I) were obtained from Vector Labs., Burlingame, CA, USA. Concanavalin A-Sepharose (Con A) was from Pharmacia (Uppsala, Sweden) and serotonin-Sepharose (SER) was a gift from Dr. R.J. Sturgeon (Edinburgh) [10]. Neuraminidase type V from *Clostridium perfringens* and β-galactosidase grade VII from Jack beans were from Sigma (Poole, UK). Activity of β-hexosaminidase was assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (4-MU-GlcNAc; Koch-Light, Colnbrook, UK), as previously described [4, 5]. Protein was measured [11] with bovine serum albumin (Sigma) as standard.

Cell Culture

CCRF/CEM and SM1 lymphoblasts were kindly provided by Dr. C.M. Steel (M.R.C. Clinical and Population Cytogenetics Unit, Edinburgh) and were grown either as a suspension or monolayer in MOPS/L medium containing 5% fetal calf serum [12]. Human skin fibroblasts were cultured as described [13] in Hams F10 medium containing 20% newborn calf serum. Cells were collected by trypsinisation or centrifugation, washed twice with phosphate buffered saline (Oxoid, Basingstoke, UK) and stored as pellets at -40°C until required.

For preparation of precursor β-hexosaminidase, cells were grown in the presence of heat-denatured serum (55°C for 60 min, to destroy endogenous enzyme activity) in medium supplemented with 10 mM NH_4Cl. For fibroblast cultures, medium was removed after 48 h and stored at -40°C until required. For lymphoblast cultures, the volume of medium was halved during NH_4Cl treatment and following removal of the medium after 24 h, cells were returned to normal culture for one week prior to further treatment.

Enzyme Preparations

For mature β-hexosaminidase, CCRF/CEM, SM1 and fibroblast pellets (3-5 mg protein) were sonicated (two 15 s bursts) in 1 ml 12.5 mM sodium succinate buffer pH 6.0 containing 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$ and 0.1% sodium azide (Con A buffer),

and after centrifugation for 5 min at 8 500 × g, supernatant fractions were applied to Con A-Sepharose.

For precursor β-hexosaminidase, culture medium was mixed with Con A buffer (ten-fold concentration) to give the correct final strength, and applied directly to Con A-Sepharose. Sufficient medium was used to provide at least 20 000 U of β-hexosaminidase (1 U will hydrolyse 1 μmol 4-MU-GlcNAc/h at 37°C). Subsequent lectin chromatography was performed using the precursor or mature β-hexosaminidase which was eluted from Con A with 0.5 M α-methyl-D-mannoside, after dialysis against the appropriate lectin buffer. When running lectin columns, amounts of protein applied were always well below the binding capacities, and enzyme activity was recovered quantitatively. For all studies, the amount of β-hexosaminidase applied to the columns (except Con A), whether purified or in crude preparation, was approximately 3 000 U.

Isoelectric Focusing

Isoelectric focusing was carried out in "J" tubes of 10 ml working capacity [4, 5] using a linear sucrose gradient (0-40% w/v) containing 1% (w/v) Ampholine® (LKB Instruments, Croydon, UK) pH range 3.5-10 and 0.1% (w/v) Triton X-100. Cell extract or dialysed medium from ammonium chloride treated cells, containing at least 3 000 U of β-hexosaminidase activity was applied to the columns and electrofocusing was performed at 400 V for 18 h at 4°C. Fractions (0.25 ml) were collected and the pH at 4°C was immediately recorded, prior to assaying enzyme activity as described earlier.

Lectin Binding Studies

Details of buffers for equilibration of immobilised lectins and elution of bound materials are given in Table 1. Columns were prepared in 2, 5 or 10 ml disposable plastic syringes and run at 21°C except SER (4°C), at a flow rate of 6 ml/h. For Con A, ten 5 ml fractions were collected with equilibration buffer and after a wash with the same buffer plus 0.5 M NaCl (ten 5 ml fractions), bound material was eluted in twenty 5 ml fractions.

For all other columns, ten 3 ml fractions were collected with equilibration buffer followed by ten 3 ml fractions with elution buffer. All eluted fractions containing buffers of molarity 0.1 M or monosaccharides were dialysed against water (5 l for 18 h) prior to assay.

Treatment with Neuraminidase and β-Galactosidase

CCRF/CEM β-hexosaminidase was purified by a rapid one-step procedure using an affinity ligand of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (Koch-Light) coupled to CNBr-activated Sepharose-4B (Sigma) [14]. Purified enzyme was divided into four aliquots (approx. 10 000 U each). To three aliquots was added an equal volume of 0.04 M sodium phosphate/0.02 M sodium citrate buffer pH 5.5 containing neuraminidase (2 mg/ml). Bovine serum albumin (2 mg/ml) replaced neuraminidase in the fourth aliquot, and this served as a control. After 4 h at 37°C, one sample and the control were each divided into three, dialysed against appropriate lectin buffers and applied to the lectin columns.

Table 1. Lectin binding studies. Details of equilibration and elution.

Lectin	Principal binding sugar(s)	Equilibrating buffer	Eluting buffer
Con A (10 ml)	mannose, glucose	12.5 mM succinate pH 6.0, 0.5 mM $MgCl_2$ 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$, 0.1% NaN_3	10 mM phosphate pH 7.0 + 0.5 M α-methyl-D-mannoside
RCA-I (2 ml)	galactose	10 mM HEPES pH 7.1, 0.15 M NaCl, 0.04% NaN_3	Equilibration buffer + 0.2 M Gal
WGA (2 ml)	*N*-acetylglucosamine	10 mM HEPES pH 7.5, 0.15 M NaCl 0.04% NaN_3	Equilibration buffer + 0.2 M GlcNAc
PHA-E (2 ml)	Gal-GlcNAc-(Man)	As for Con A	Equilibration buffer + 1 M NaCl
SER (5 ml)	*N*-acetylneuraminic acid	H_2O	0.2 M phosphate pH 7.0 + 0.5 M NaCl

The conditions for running the columns are given in the Materials and Methods section.
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

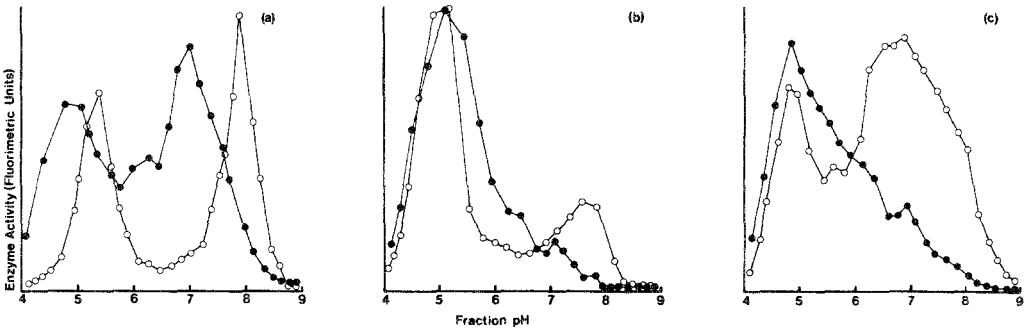

Figure 1. Isoelectric focusing profiles of precursor (●) and mature (○) β-hexosaminidase in (a) fibroblasts, (b) SM1 lymphoblasts, and (c) CCRF/CEM lymphoblasts.

To one of the remaining aliquots was added an equal volume of 0.1 M sodium citrate buffer pH 4.0 containing 0.2 U of β-galactosidase, while buffer alone was added to the other to act as a control. After 24 h at 37°C, the samples were each divided into three, dialysed against appropriate lectin buffers and applied to the lectin columns.

Results

Isoelectric Focusing

The isoelectric focusing profiles of precursor and mature β-hexosaminidases are shown in Fig. 1. Fibroblast mature β-hexosaminidase activity was resolved into two major isoenzymes, A and B, focusing at pH 5.2 and 7.9, respectively. In contrast, the precursor enzyme exhibited peaks at pH 4.9 and 7.1 with a minor intermediate at pH 6.3. Both SM1 and CCRF/CEM precursor β-hexosaminidase activity profiles had a major peak at pH 5.0 with smaller peaks at pH 6.3 and 7.0. The focusing profile of the mature SM1 enzyme exhibited peaks at pH 5.1 and 7.8 corresponding to A and B respectively, while CCRF/CEM mature β-hexosaminidase had peaks at pH 4.9 and 7.0 with minor peaks at pH 6.3 and 7.8. Specific activities of β-hexosaminidase in the cells and culture medium studied are given in Table 2.

Lectin Binding Studies

Results of lectin binding studies are shown in Fig. 2. All enzymes showed approximately 100% binding to Con A and this fraction was used in all subsequent binding studies.

(a) *Fibroblast β-hexosaminidase.* Precursor and mature β-hexosaminidase exhibited similar degrees of binding to PHA-E with 50% and 60% binding, respectively. Affinity for WGA was greater for precursor β-hexosaminidase with 50% binding while only 25% of the mature form bound to this lectin. However, the level of binding to SER was greater

Table 2. Activity of β-hexosaminidase in cell and medium preparations.

	Cellular β-hexosaminidase activity ($U \times 10^{-4}$ per mg protein)[a]	Medium β-hexosaminidase activity[b] (U/ml medium)
CCRF/CEM Lymphoblasts	223.2 ± 16.8 (n = 3)	2.1-4.2
SM1 Lymphoblasts	201.6 ± 48.0 (n = 3)	10-15
Control fibroblasts	427.8 ± 57.0 (n = 10)	10-15

[a] 1 Unit = 1 μmol 4-methylumbelliferone liberated/h.

[b] Enzyme activities measured in culture medium were variable and dependent on factors such as volume of medium, number of cells and their growth characteristics, and time in the presence of NH_4Cl. The units shown give a range of values found in these studies.

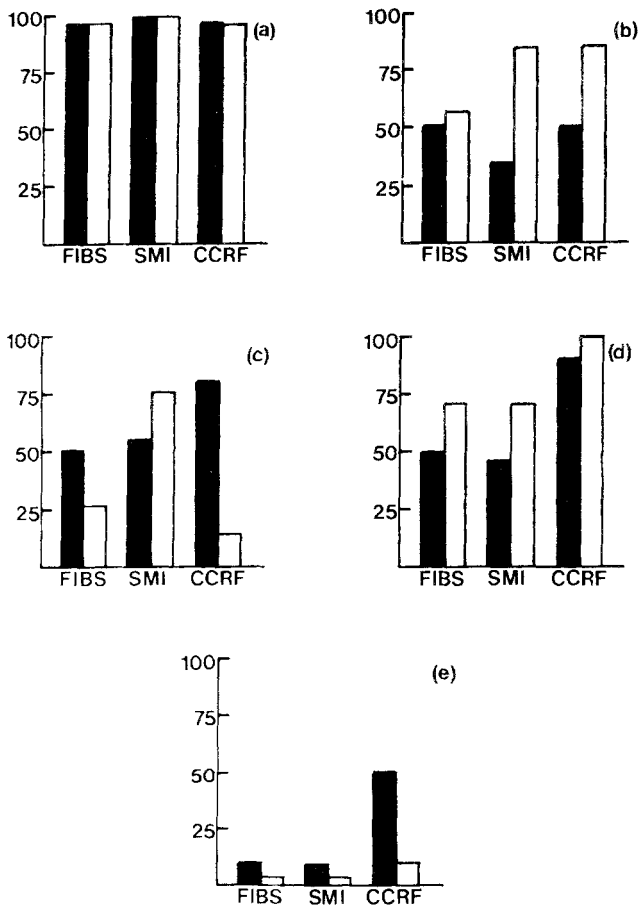

Figure 2. Binding of β-hexosaminidase to immobilised lectins. (a) Con A, (b) PHA-E, (c) WGA, (d) SER, (e) RCA-I. Columns shows % total activity which bound for FIBS (control fibroblasts), SM1 (normal lymphoblasts) and CCRF (acute leukaemic lymphoblasts). Closed columns, precursor enzyme. Open columns, mature enzyme.

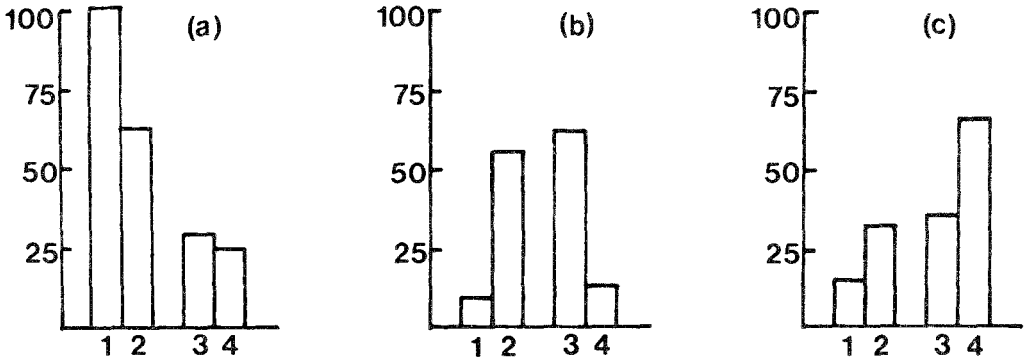

Figure 3. Effects of *exo*glycosidase treatments on binding of CCRF β-hexosaminidase to immobilised lectins. Columns show % total activity. Samples were either treated for 4 h in the absence (1), or presence (2) of neuraminidase; or having received 4 h neuraminidase treatment, a further 24 h in the absence (3), or presence (4) of β-galactosidase. Lectins used were (a) SER, (b) RCA, (c) WGA.

for the mature enzyme (70%) than the precursor (50%) while binding to RCA-I was low for both precursor and mature forms at 10% and 5%, respectively.

(b) *SM1 β-hexosaminidase.* Affinity of SM1 β-hexosaminidase for PHA-E was considerably higher in the mature form than in the precursor with 85% and 35% binding, respectively. The binding patterns to WGA and SER were virtually identical with 50% precursor activity binding compared with 75% for mature enzyme. The binding pattern to RCA-I was similar to that of fibroblast β-hexosaminidase, with low lectin affinity for both precursor and mature forms at 10% and 5%, respectively.

(c) *CCRF/CEM β-hexosaminidase.* Enzyme from CCRF/CEM lymphoblasts exhibited higher binding to PHA-E for the mature form than the precursor form with 85% and 50% bound, respectively; whereas binding to WGA was higher for the precursor form than the mature form with 80% and 15% bound, respectively. High affinities for SER were observed with both precursor (90% bound) and mature (100% bound) forms. Binding to RCA-I was considerably higher for the precursor enzyme at 50% than the mature enzyme at 10%.

Exoglycosidase Treatments

Results of neuraminidase and β-galactosidase treatments on lectin affinities of CCRF/CEM mature β-hexosaminidase are given in Fig. 3. Incubation of ligand-purified β-hexosaminidase in the absence of neuraminidase showed that the lectin binding characteristics were identical to those of the enzyme in the crude extract, with near total binding to SER, 10% binding to RCA-I, and 15% binding to WGA. After 4 h neuraminidase treatment, the affinities changed, with binding to SER reduced from approximately 100% to 65%, binding to RCA-I elevated from 10% to 55% and binding to WGA raised from 15% to 30%. Prolonged treatment with neuraminidase (24 h) in the absence of β-galactosidase extended these changes with binding to SER further reduced to 30%, binding to RCA-I increased to 65% and binding to WGA raised to 35%. Prolonged neura-

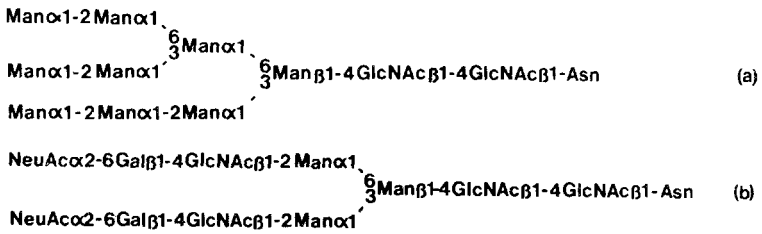

Figure 4. Structures of asparagine-linked oligosaccharides. (a) High-mannose and (b) bi-antennary complex. For review see [33].

minidase treatment combined with β-galactosidase treatment had little further effect on binding to SER, whereas binding to RCA-I was reduced to 12%, and binding to WGA increased to 70%.

During these *exo*glycosidase treatments, there was no significant loss of β-hexosaminidase activity, and total recoveries were obtained from lectin columns.

Discussion

The binding patterns of fibroblast β-hexosaminidase to Con A, RCA and WGA are similar to those reported elsewhere [15, 16] for these cells and for human liver [8], and are believed to be consistent with the presence of both oligomannosidic and complex-type *N*-linked glycans on the enzyme molecule (Fig. 4) [17-19]. The finding that CCRF/CEM β-hexosaminidase exhibits a markedly different pattern of lectin affinities suggests that a different type of enzyme glycosylation exists in these cells. The control lymphoblastoid cell-line, SM1, displays β-hexosaminidase lectin-binding patterns (with the exception of WGA) similar to those of fibroblasts, further supporting the evidence that the aberrant glycosylation of β-hexosaminidase in CCRF/CEM cells may not be due to their transformation *per se*, but a reflection of their malignant state. The results of binding to PHA-E must be interpreted with care, because although affinity for this lectin is usually associated with complex oligosaccharides possessing a bisecting *N*-acetylglucosamine [20], it is believed that hydrophobic interactions between the lectin and aglycone regions of glycoproteins may also result in binding [21].

The pattern of β-hexosaminidase binding to RCA and SER may be more reliably interpreted in terms of oligosaccharide structure. Enzyme from all three cell types showed an increase in affinity for SER during maturation, with a coincident decrease in binding to RCA. CCRF/CEM β-hexosaminidase differs from SM1 and fibroblast enzymes in exhibiting appreciably increased affinities for RCA and SER in the precursor and mature forms respectively. It appears, therefore, that in the processing of these complex oligosaccharides, terminal galactose residues evident in the precursor form of β-hexosamini-

dase become sialylated in the mature lysosomal form. In this context, CCRF/CEM β-hexosaminidase differs from the fibroblast and SM1 enzyme, in possessing more terminal galactose in its precursor form, and subsequently more sialic acid in the mature form.

These observations were confirmed by *exo*glycosidase treatments on CCRF/CEM β-hexosaminidase. Neuraminidase treatment suppressed binding to SER, whilst increasing affinity for RCA. Neuraminidase treatment combined with β-galactosidase treatment resulted in low affinities for SER and RCA, but an elevated degree of binding to WGA. This strongly suggests the sequence terminal NeuAc-Gal-GlcNAc which is commonly found in complex type oligosaccharides (Fig. 4). The isoelectric focusing profiles provide further evidence for hypersialylation of CCRF/CEM β-hexosaminidase as suggested earlier [4] for acute lymphoblastic leukaemic cells. Both SM1 and fibroblast mature β-hexosaminidase focuses as two main peaks, A and B, at pH 5.2 and 7.9, respectively. In CCRF/CEM cells, the isoenzymes focus at pH 4.9 and 7.1, and the existence of these anodic forms would be consistent with an increased molecular electronegativity caused by hypersialylation. Fibroblast precursor β-hexosaminidase also electrofocuses with forms at pH 4.7 and 7.1 but since binding to SER is not high, this may be due to negatively charged moieties other than sialic acid, such as mannose-linked phosphate groups, which exist on fibroblast precursor lysosomal enzymes [19].

Although abnormal β-hexosaminidases have been reported in several human malignancies including lung cancer [22] and liver metastases [23], only in leukaemia has abnormal glycosylation been suggested as the possible cause. That the abnormality should be manifested as hypersialylation is of particular interest since this condition is more usually associated with serum forms of lysosomal enzymes. In serum, the high level of sialylation serves a functional role by protecting lysosomal enzymes from endocytosis, but our investigations have failed to find any reasons why intracellular β-hexosaminidase should be hypersialylated, while activity, thermostability, K_M and pH optima are all unaffected (S.E. Moss, unpublished data).

The increased level of sialylation of β-hexosaminidase in CCRF/CEM lymphoblasts may be the consequence of an increased availability of terminal galactose in the precursor form, as demonstrated by a high affinity for RCA (Fig. 2e). The actual defect may occur early in the processing of these oligosaccharide chains, where a small change in the core portion of the glycan may result in subsequent structural changes. Precursor β-hexosaminidase secreted from mucolipidosis II (I-cell disease) fibroblasts exhibits a similarly increased affinity for RCA [15, 24]. In I-cell disease, an inherited genetic disorder of oligosaccharide processing resulting in the extracellular accumulation of lysosomal enzymes, secreted β-hexosaminidase also exhibits hypersialylation [25]. The elevated levels of terminal galactose and sialic acid are coincident with a decreased affinity of I-cell β-hexosaminidase for Con A [26] and have been partly attributed to a conversion of oligomannoside chains to complex chains [27]. However, in CCRF/CEM lymphoblasts, binding of β-hexosaminidase to Con A remains high, so the increased presence of terminal galactose in the precursor form and sialic acid in the mature form is probably not due to extra processing of high-mannose chains.

The processing of complex oligosaccharides of lysosomal enzymes is controlled by the combined activities of glycosyltransferases and both lysosomal and non-lysosomal gly-

cosidases. Elevated levels of galactosyl- and sialyltransferases have been found in acute leukaemic lymphoblastoid cell-lines [28] and lysosomal sialidase and β-galactosidase have been demonstrated as having roles in determining the structures of β-hexosaminidase oligosaccharides [16]. A Chinese hamster ovary cell-line has been recently reported [29] in which a dominant mutation induces a specific glycosyltransferase, resulting in abnormal oligosaccharide biosynthesis. A similar defect in leukaemic cells could account for the abnormal glycosylation reported here. Therefore, whilst there is strong evidence for the presence of high-mannose type and complex-type chains, also reported for β-hexosaminidase from bovine brain [30], human liver [17, 18] and fibroblasts [19], the co-existence of hybrid, tri-antennary or tetra-antennary chains remains a possibility. The abnormal occurrence of such glycans has been reported in several malignancy-associated glycoproteins [31, 32] and further investigation into the effect of malignancy on β-hexosaminidase oligosaccharides is in progress.

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