Acta Biochimica Polonica

Vol. 45 No. 2/1998

361–371

QUARTERLY

# Contents of total and protein-bound carbohydrates are low in leukemic leukocytes from patients with acute myelogenous leukemia*

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**Key words**: myelogenous leukemia, carbohydrates, protein-bound carbohydrates, ceramide-bound carbohydrates

**Leukemic leukocytes from 12 patients with acute myelogenous leukemia (AML) and two patients with chronic myelogenous leukemia (CML) were isolated by centrifugations in Percoll gradients, and examined for total carbohydrates. In leukemic leukocytes from 10 of these patients ceramide-bound carbohydrates were also determined. Protein-bound carbohydrates were calculated by subtraction of ceramide-bound carbohydrates from total carbohydrates. In all samples analysed the contents of total and protein-bound carbohydrates were much lower in leukemic leukocytes than in normal neutrophils, irrespective whether the results were expressed relative to protein, DNA, cell number or dry mass. For immature leukemic cells of MO-M1 phenotype differences up to 10-fold were observed. Contents of ceramide-bound carbohydrates, i.e. those of neutral and acidic glycosphingolipids (GSLs) were also low in leukemic cells. However, when GSL carbohydrates were calculated as percentage of total carbohydrates, GSLs in leukemic leukocytes were elevated in half of the AML patients but depressed in the other half. The results are discussed in the light of the hypothesis on GSL function by one of us (Kościelak J., 1986, *Glycoconjugate J.* 3, 95–108). According to one element of the hypothesis, during cell differentiation newly synthesized glycoproteins (GPs) that perform specific functions are added to house-keeping GPs that are present in plasma membranes of all types of cells. Thus, during differentiation, the GP content of the cell membrane should increase and that of the so called "membrane packing" glycosphingolipids should decrease.**

*Presented at the International Symposium on Structure, Metabolism, and Functions of Glycosphingolipids and Sphingolipids in Warsaw, Poland, September 4–6, 1997.

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**Abbreviations:** AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; $CML_{BC}$, CML blast crisis; GSL(s), glycosphingolipid(s); GP(s), glycoprotein(s); PG(s) proteoglycan(s), D-PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; L-PDMP, L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; CPDA-1, citrate-phosphate-dextrose-adenine; ACD, acid-citrate-dextrose

In all living cells, plasma membrane carbohydrates are bound to either proteins or lipids. In mammalian cells, lipid-bound carbohydrates are mostly glycosphingolipids (GSLs). Whereas the role of carbohydrates in glycoproteins (GPs) and proteoglycans has been subject to numerous investigations and largely resolved, the significance of lipid glycosylation is less clear. One of us (Kościelak, 1986a, b) has proposed that in the plasma membrane, some GSLs are „membrane packing substances" and provide an energetically cheap and largely inert carbohydrate protective coat. This was to be the „basic" function of all GSLs. Specific functions for GSLs were envisaged among others for gangliosides, certain fucoglycosphingolipids, and GSLs terminated with β-galactopyranosyl residues. According to the present knowledge specific functions mediated by GSLs are: cellular differentiation (Nojiri *et al.*, 1988), adhesion, (Stroud *et al.*, 1995; Taki *et al.*, 1997), proliferation (Bhunia *et al.*, 1996; reviewed by Inokuchi, 1997), modulation of functions of receptors for PDGF and EGF, signal transduction, and homo- and heterotype carbohydrate– arbohydrate interactions (reviewed by Hakomori, 1990), induction of specific kinases at the plasma mebrane of nerve cells and promotion of neurite outgrowth (reviewed by Nagai, 1995), synapse formation and memory retention (Inokuchi *et al.*, 1998). GSLs are also receptors for bacteriae and viruses (Karlsson, 1998) but in most instances this is not a physiological but, as we call it, renegade function. The discovery of specific inhibitors (D-PDMP, fumonisin, N-alkylated imino sugars) and activators (L-PDMP) of GSL biosynthesis (reviewed by Radin & Inokuchi, 1991, Platt & Butters, 1995; Inokuchi, 1997) has provided investigators with tools for studying directly the function of GSL. In cell culture, inhibition of the biosynthesis of GSLs by D-PDMP results, among others, in growth arrest and change of cell morphology. The compound may be toxic when administered to whole animals. Medaka fish embryos, however, hatched normally in the presence of D-PDMP in spite of the apparent absence of GSLs from their tissues (Fenderson *et al.*, 1992). The question „do we really need glycosphingolipids ?" was posed by one of the authors of that study (Radin, 1992). This is an important question as underscored by the subsequent finding that GSLs-deficient mouse melanoma cells are able to survive and proliferate *in vitro* (Ichikawa *et al.*, 1994).

During cell differentiation newly synthesized GPs that perform specific functions are probably added to house-keeping GPs that are present in plasma membrane of all types of cells (Kościelak 1986a, b). Thus, it was hypothesized that, during differentiation, the GP content of the cell membrane should increase and that of „the membrane packing GSLs" decrease. A possible inverse relation between GPs and GSLs at the cell membrane has been also suggested on the basis of a putative increase of GSLs contents in tumours (reviewed by Hakomori, 1996) and temporal difference in GSL and GP biosynthesis during the cell cycle (Chatterjee *et al.*, 1975a, b; Pudymaitis & Lingwood, 1992). Thus, the biosynthesis of simple GSLs occurs predominantly in the mitosis and G1 phase of the cell cycle (Chatterjee, 1975a; Collins & Warren, 1992), whereas GPs are synthesized late in the S phase. In the latter phase, however, GSLs are hydrolysed (Chatterjee, 1975b). Absence of GP biosyntesis during mitosis may be due to fragmentation of Golgi apparatus into small vesicles (reviewed by Warren, 1992).

In the present study we put some of the afore mentioned ideas to an experimental test by measuring total and ceramide-bound carbohydrates in leukemic cells. The cells representing different differentiation stages, were obtained from yet untreated patients with acute and chronic myelogenous leukemia (AML and CML, respectively). Results were expressed relative to protein, DNA, cell number, and dry mass.

## MATERIALS AND METHODS

***Patients.*** The study involved 12 patients with AML and two patients with CML. Among 12 AML patients, 10 were new cases while two patients were studied during a blast crisis that occurred in the course of CML. To secure a sufficient amount of material for analysis, patients were selected for high counts of leukemic cells. The disease subtype was classified according to FAB (French-American-British) criteria, and immunophenotypes of leukemic cells. All patients except one were not treated with cytostatic drugs. The treated patient (Ka) received 5 daily doses of 2.0 g of hydroxycarbamide prior to blood collection.

***Isolation of normal and leukemic cells.*** Normal cells from healthy donors were isolated from buffy coats prepared by centrifugation at 5000 × *g* for 5 min at 4°C of freshly drawn blood portions (450 ml) anticoagulated with 63 ml of CPDA-1. Blood of leukemic patients was collected on 1/10 vol. of ACD and thereafter directly fractionated omitting buffy coat isolation. Fractionation of either the buffy coat from normal blood or whole blood from leukemic patients was performed by centrifugation of samples on a self-generated gradient of Percoll by the method of Pertoft *et al.* (1979). In the case of normal white cells, the method allowed to separate lower band containing pure neutrophils and an upper band that was a mixture of lymphocytes and monocytes. Lymphocytes and monocytes were subsequently separated from each other by a second centrifugation in an angle rotor on a different Percoll gradient prepared according to Gmelig-Meyling & Waldmann (1980). Leukemic leukocytes banded in the method of Pertoft *et al.* (1979) together with lymphocytes and monocytes and were analysed as such without further purification.

***Cell counting.*** Cell concentrations were determined using a Cell-Dyn 1500 Hematology Analyser made by Sequoia-Turner (U.S.A).

***Determination of cell immunophenotypes.*** Immunophenotypes of normal and leukemic blood cells were determined by flow cytometry employing monoclonal antibodies (mAbs). Binding of mAbs to cells was assessed by direct immunofluorescence using appropriate single and double staining combinations with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). For single staining the following mAbs were used: CD34 (PE) (Becton Dickinson), HLA-DR (PE) (Becton Dickinson), and CD71 (FITC) (DAKO). The mAbs used in combinations were: CD45/CD14 (DAKO), CD13/CD33 (DAKO), CD15 (DAKO)/CD117 (IMMUNOTECH). Other mAbs used were: CD7 (FITC) (DAKO), CD56 (PE) (Becton Dickinson), CD16 (PE) (DAKO), CD66 (RPE-Cy5, i.e. PE-cyanin 5) (DAKO).

Flow cytometry was performed on a Cytoron Absolute instrument (Ortho) by collecting 10000 ungated events. Selection of an appropriate leukemic cell gate was based on both forward (FW-SC) and right scatter (RT-SC), as well as CD45 expression and RT-SC. The latter allowed differentiation between mature and immature cells. Thresholds for positivity were based on isotype negative controls. Positivity was defined as brighter staining intensity than that of isotype controls in more than 20% of cells. Data acquisition and analysis were performed using IMMUNOCOUNT II (Ortho) and WinMDI 2.1.4. software programs, respectively.

***Isolations of GSLs.*** Cells were washed with phosphate-buffered saline (pH 7.4), lyophilized, and kept at −40°C before use. The lyophilized material, equivalent to 2–20 × $10^8$ viable cells was sequentially extracted with chloroform/methanol/water (1:1:0.2, by vol); chloroform/methanol (2:1; v/v), chloroform/methanol (1:2 v/v); and propanol/hexane/water (55:25:20, by vol.). Each extraction lasted 30 min at room temperature. The extracts were combined, desalted, acetylated, and thereafter processed as described by Zdebska *et al.* (1996). Briefly, acetylated GSLs were separated from phospholipids using Florisil column chromatography (Saito & Hakomori, 1971). Fractions containing GSLs

were collected, deacetylated, and separated into neutral and acidic GSLs by DEAE-Sephadex ion exchange chromatography. Neutral and acidic GSLs were subsequently purified by silicic column chromatography according to Saito & Hakomori (1971) and Lee *et al.* (1982b), respectively.

***Chemical analyses.*** Protein was determined by the method of Lowry *et al.* (1951) but in the presence of sodium dodecyl sulfate (BioRad modification). Sphingosine was determined according to Higgins (1984).

***DNA determination.*** DNA was determined according to the method described by Ormerod (1990) as modified by us employing Perkin-Elmer spectrometer LS 50. Briefly, cells were digested first with proteinase K (Merck) in 0.012% sodium dodecyl sulfate for 24 h at 37°C, and then with DNase-free RNase (Sigma) for 30 min at 37°C. After addition of ethidium bromide, readings were made at 611 nm using excitation at 257 nm.

***Carbohydrate determination.*** The analysis of carbohydrates was performed by High pH Anion Exchange Chromatography as described by Zdebska *et al.* (1996).

Total carbohydrates were determined in hydrolysates of whole, lyophilized cells. GSL carbohydrates were determined in hydrolysates of neutral and acidic GSLs. Protein-bound carbohydrates were calculated by subtracting GSL carbohydrates from total carbohydrates.

## RESULTS

### Normal and leukemic cells

The isolation methods used, allowed to obtain purified preparations of normal neutrophils, lymphocytes and monocytes. According to flow cytometry, neutrophils were contaminated with about 1% of lymphocytes, lymphocytes contained about 5% of monocytes, and monocytes about of 8% lymphocytes.

Leukemic cells banded in Percoll gradients together with lymphocytes and monocytes (see Fig.1a). Cells of this band were resolved, however, into distinct populations by flow cytometry. This enabled us to calculate the percentage of contaminating cells (see Table 1). Leukemic cell preparations were reasonably pure except those from patients Kr, Gr, and Ch. In addition to leukemic cells we were able to isolate normal neutrophils from three patients (Gr, Ka, and Ch). In two patients (Ka, Ch) an additional band of immature neutrophils was seen that located above normal neutrophils (see Fig. 1).

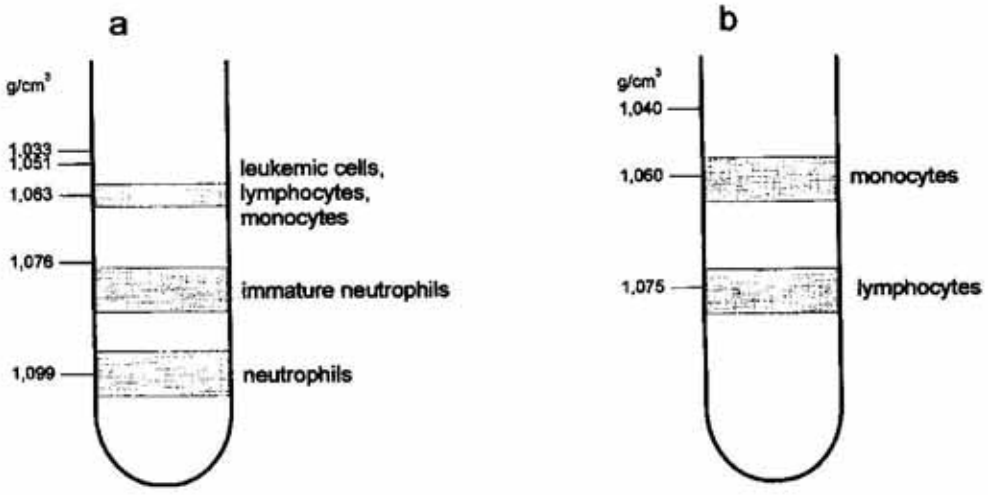


**Figure 1. Separation of blood cells by centrifugation in Percoll gradients.**

a. By the method of Pertoft *et al.* (1979); b. By the method of Gmelig-Meyling & Waldmann (1980).

**Carbohydrate contents of normal and leukemic cells**

Figure 2 shows total carbohydrate content in leukemic cells, normal neutrophils, lymphocytes, and monocytes. Glucose contents (except glucose of GSLs) were not included in calculations because they were much higher in normal than in leukemic cells (596.2 ± 82.6 nmol/100 $\mu$g DNA in normal neutrophils, but

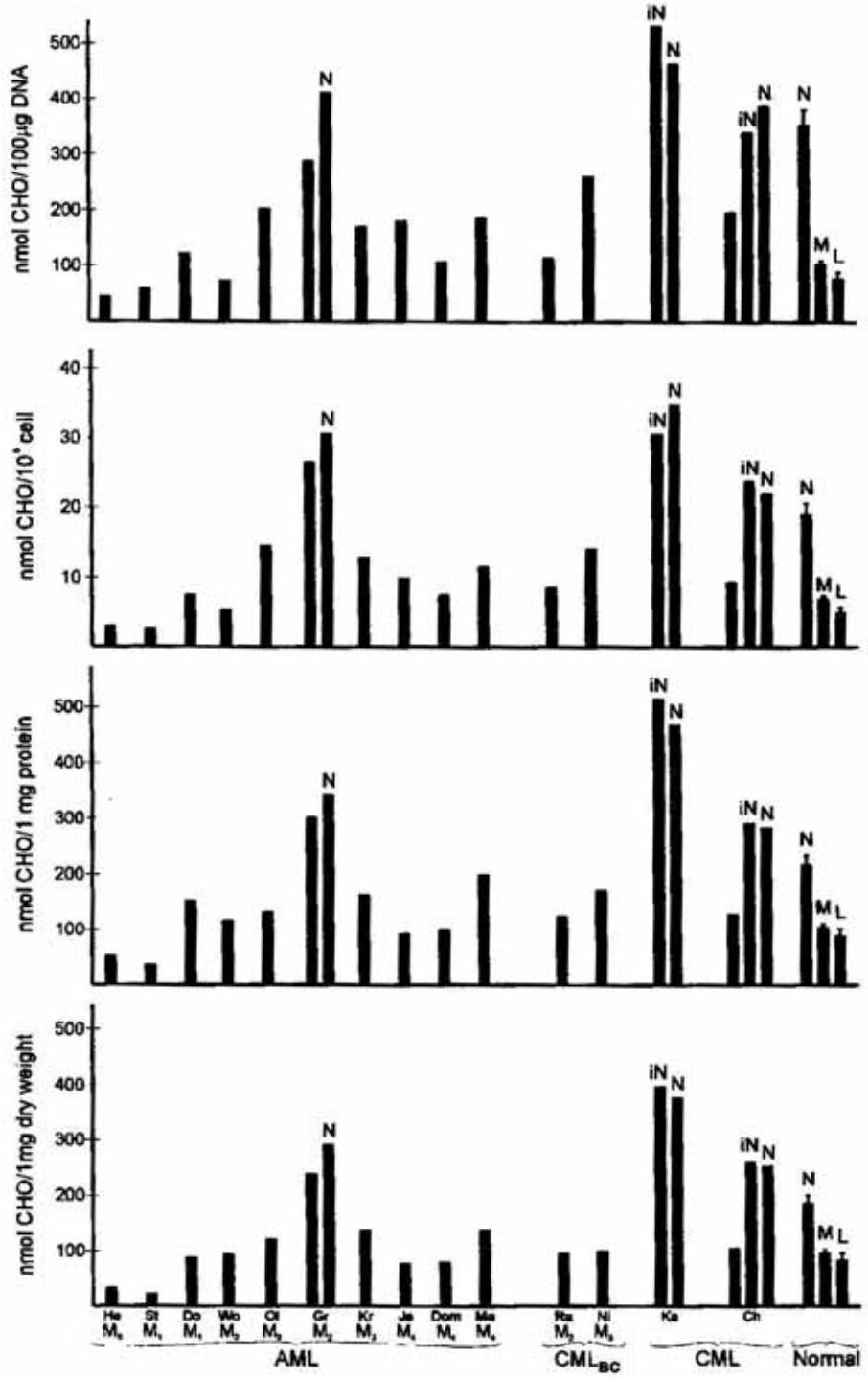


**Figure 2. Total carbohydrates in leukemic leukocytes from individual patients and control cells.**

N, neutrophils; iN, immature neutrophils; M, monocytes; L, lymphocytes.

below 50 nmol/100 $\mu$g DNA in 10 out of 12 patients with AML). This glucose was, most likely, derived from glycogen (Zdebska *et al.*, 1996) and thus would reflect cell metabolism rather than cell structure. It was evident that poorly differentiated leukemic cells of mye-

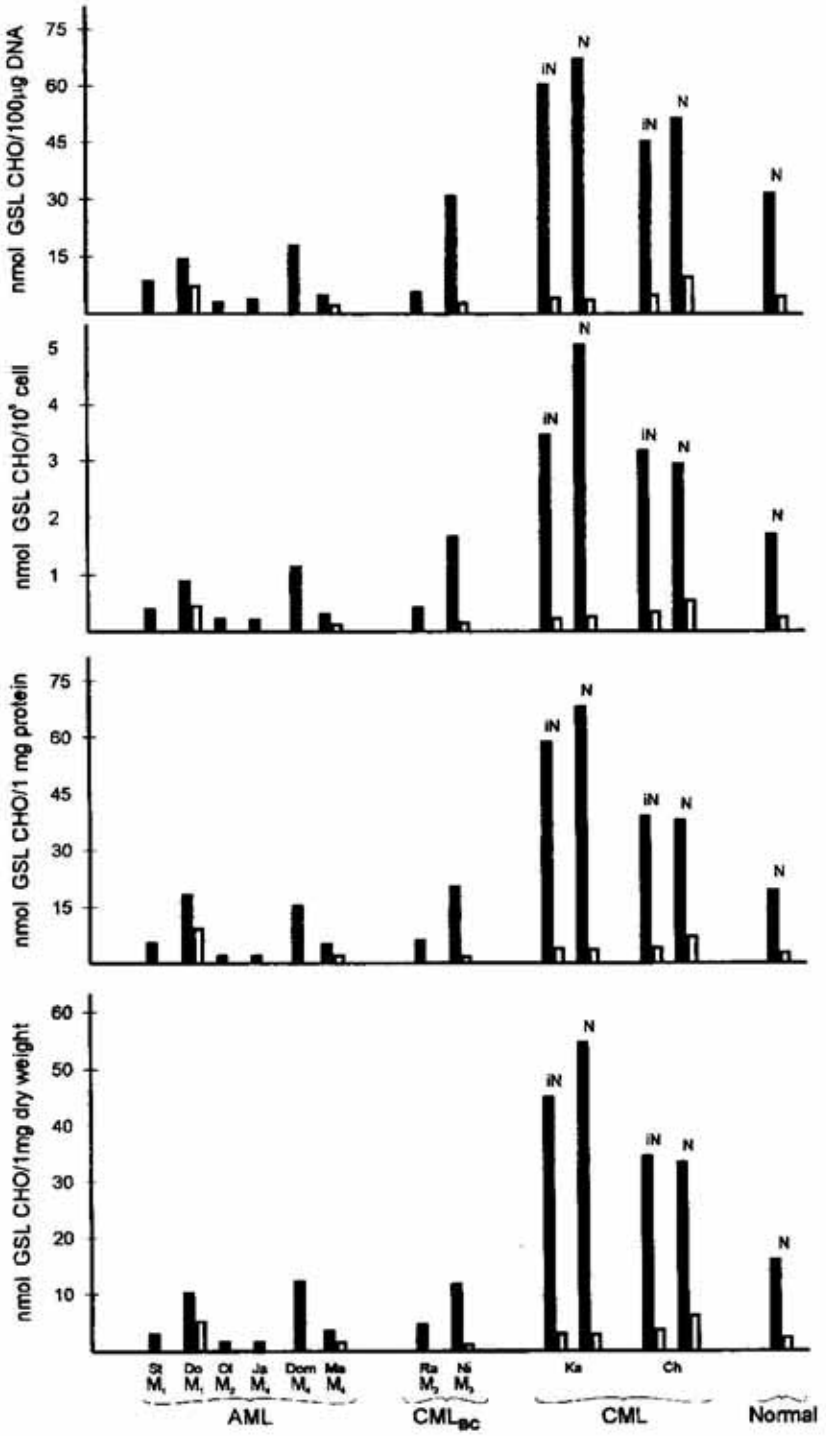


**Figure 3. Ceramide-bound carbohydrates in leukemic leukocytes from individual patients and control cells. GSL carbohydrates: filled in bars, carbohydrates of neutral GSLs; open bars, carbohydrates of acidic GSLs.**

Values for patients Gr, Kr, and Ch were corrected for lymphocytes present in leukemic cells analysed.

**Table 1. Cellular compositions and counts of white cells from leukemic patients**

| Patient | He | St | Do | Wo | Ol | Gr | Kr | Ja | Dom | Ma | Ra | Ni | Ka | Ch |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type of leukemia | AML M0 | AML M1 | AML M1 | AML M2 | AML M2 | AML M2 | AML M3 | AML M4 | AML M4 | AML M4 | $CML_{BC}$ M3 | $CML_{BC}$ M2 | CML | CML |
| Leukocyte composition in blood (%) | | | | | | | | | | | | | | |
| Undifferentiated blasts | 94 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Myeloblasts | - | 82 | 79 | 97 | 59 | 18 | 29 | 75 | 41 | 9 | 74 | 10 | 1 | 5 |
| Promyelocytes | - | - | 14 | - | - | - | 11 | - | - | - | 19 | 60 | 2 | - |
| Monoblasts | - | - | - | - | - | - | - | 9 | 17 | 71 | - | - | - | - |
| Monocytes | - | - | - | - | 3 | - | 1 | - | - | - | 0 | 7 | - | 2 |
| Lymphocytes | 4 | 14 | 2 | 1 | 2 | 16 | 13 | 4 | 2 | 6 | 1 | 1 | 3 | 4 |
| Neutrophils | - | - | - | - | 24 | 60 | 46 | - | 24 | 10 | - | - | 94 | 79 |
| Cells analyzed (count × $10^6$) | | | | | | | | | | | | | | |
| Leukemic | 13 | 296 | 248 | 1147 | 894 | 9 | 34 | 880 | 426 | 767 | 1950 | 543 | - | 140 |
| Immature neutrophils | | | | | | | | | | | | | 1520 | 308 |
| Neutrophils | | | | | | 25 | | | | | | | 690 | 292 |
| Lymphocyte contamination in leukemic cells (%) | 4 | 15 | 2 | 1 | 3 | 47 | 32 | 5 | 2 | 6 | 1 | 1 | - | 36 |

loid lineage i.e. belonging to MO-M2 phenotype, had significantly less of carbohydrates than control neutrophils irrespective whether the results were related to cell number, dry mass, or DNA or protein contents. In the case of patient Gr, apart from leukemic cells, we isolated and analysed his normal neutrophils. The latter had 409 nmol of carbohydrates/100 $\mu$g DNA, i.e. more than leukemic leukocytes that had only 271.9 nmol carbohydrate/100 $\mu$g DNA (value corrected upwards for the presence of lymphocytes in leukemic cells; see legend to Fig. 1 and Table 1). Leukemic cells from four patients with monocytic type of AML (Kr, Dom, Ja, and Ma) contained significant numbers of myeloblasts or promyelocytes. In only one of them (Ma) counts of leukemic cells of monocytic lineage exceeded those of neutrophil lineage. These cells contained more carbohydrates (185 nmol/100 $\mu$g DNA) than did control monocytes (139.8 nmol/100 $\mu$g DNA) but less than did control neutrophils.

We also studied two patients with CML (Ch and Ka). We were able to isolate and analyse three populations of cells from patient Ch: the lightest and least differentiated fraction of leukemic leukocytes (CD16–; CD66+) exhibited a carbohydrate content of 212.0 nmol/100 $\mu$g DNA; the intermediate fraction of immature neutrophils (CD16+; CD66+) had a carbohydrate content of 338.2 nmol/100 $\mu$g DNA, while normal neutrophils (CD16++; CD66+) had 386.3 nmol of carbohydrates/100 $\mu$g DNA. In patient Ka, both immature and normal neutrophils exhibited higher than normal contents of carbohydrates.

### Glycosphingolipids

GSL contents in normal and leukemic cells are shown in Fig. 3. Contents of GSL carbohydrates in leukemic leukocytes from patients with AML (calculated in nmol per 1 mg protein or 1 mg dry weight) were lower than in normal neutrophils though in three patients the differences were small. When GSL carbo-

hydrates were expressed as percentage of total carbohydrates, leukemic cells of some patients (St, Do, Dom, Ni) exhibited, however, higher than normal values (14.9%, 17.9%, 15.4%, 13.0.%, respectively; control neutrophils had 10.1.% GSL carbohydrates). In other four patients with AML (Ol, Ja, Ma and Ra) the content of GSL carbohydrates in leukemic leukocytes was low even when expressed as % of total carbohydrates (1.4%, 2.0.%, 3.7%, 4.9.%, respectively). Neutrophils and immature neutrophils from two patients with CML (Ka and Ch) had higher than normal contents of GSL carbohydrates both in terms of nmol per 1 mg of protein dry mass or relative values (12.1% and 14.8% for immature neutrophils; 14.8% and 15.8% for neutrophils). The amounts of acidic glycosphingolipids varied but the mean value was close to normal. Carbohydrate molar ratios of fractions containing neutral and acidic GSL strongly suggested that the predominant component of the former was lactosylceramide and of the latter, $G_{M3}$. This was confirmed by TLC (not shown). Lack of material precluded a more detailed investigation of GSLs. In fact, we were even unable to isolate GSLs from all patients. We have noticed, however, that in hydrolysates of neutral GSLs from the least differentiated leukemic cells analyzed (patients St and Do), glucosamine was notably absent. We have also seen small galactosamine peaks in hydrolysates of neutral GSL from several of our patients.

The contents of protein-bound carbohydrates were calculated for leukemic leukocytes from 10 patients in which GSL carbohydrates were determined. Results (not shown) were very similar to those obtained when total carbohydrates of normal neutrophils and leukemic leukocytes were compared.

### Carbohydrate molar ratios in normal and leukemic cells

Total cell carbohydrates were quantitated by summation of contents of individual sugars present in hydrolysates of whole cells. Thus, unplanned by-products of this investigation were molar ratios of individual carbohydrates in normal and leukemic cells. For normal neutrophils these ratios were: Fuc 6.7 0.8; GalN 5.4 0.6; GlcN 31.5 1.0; Gal 30.3 1.4; Man 22.4 2.5; NeuAc 3.6 0.9. For leukemic cells the ratios were: Fuc 4.8 1.1; GalN 6.4 1.8; GlcN 30.7 5.5; Gal 23.4 5.4; Man 28.0 10.1; NeuAc 8.9 4.1. The differences in carbohydrate molar ratios between normal and leukemic cells were statistically insignificant.

## DISCUSSION

Leukemia arises from excessive proliferation of an abnormal cell clone that is unable to mature. Overproduced cells of different maturity enter blood and may be isolated therefrom in a reasonable state of purity. Thus, leukemia offers a good model for studies on quantitative relations between GSLs and GPs during maturation. A limitation of this model is, however, a superimposition of features caused by maturation by those due to neoplasia.

The main conclusion of this study is that immature cells of myeloid lineage from AML patients have much less of total carbohydrates than more mature cells from patients with CML or normal neutrophils. Differences may be as large as tenfold and were observed also between leukemic and normal cells isolated from the same patient. Furthermore, the differences were not associated with cell size as they were clearly detected when total cell carbohydrates were calculated with respect to protein content and dry mass. We have also calculated protein-bound carbohydrates by subtracting ceramide-bound carbohydrates from total carbohydrates but conclusions were exactly the same. Thus, the assumption that fully mature cells should contain more protein-bound carbohydrates than immature cells (Kościelak, 1986a, b) has been confirmed. The assumption also envisaged, how-

ever, an increase of „membrane packing" GSLs, which should have been reflected in accumulation of ceramide-bound carbohydrates. We have indeed found that leukemic leukocytes from five patients had – in relation to total carbohydrates – more ceramide-bound carbohydrates than control neutrophils. In other four patients, however, immature leukemic leukocytes contained less ceramide-bound carbohydrates than normal neutrophils. It is possible that these divergent results were caused by the neoplastic process because an abnormal number or structure of chromosomes are very common in AML, and can be detected in 50–80% of patients (Lichtman, 1995). Virtually any chromosome may be rearranged, deleted or added. The probability that contents of GPs and GSLs are deranged in leukemic cells is therefore high. It is probably higher for GSLs because aberrant biosynthesis of GPs is more likely to affect cells viability. Validity of this argument is supported by diseases of aberrant glycosylation that are caused by deranged biosynthesis of GPs or PGs but never of GSLs (Kościelak, 1995).

As to our knowledge, a quantitative study of total and ceramide-bound carbohydrates in leukemic cells has not been performed before. There is, however, a number of reports on the presence and structures of individual glycoconjugates including GSLs (e.g. Fukuda *et al.*, 1986). It was also found that leukemic leukocytes of myeloid lineage contain both „globo" and „neolacto" GSLs whereas in mature neutrophils only the latter are present (Lee *et al.*, 1982a, b; reviewed by Macher *et al.*, 1982; Buehler *et al.*, 1885). Neolacto GSLs increase in myeloid cells during maturation to neutrophils (Nojiri, *et al.*, 1988; Nakamura *et al.*, 1992; Hu *et al.*, 1994). Our results are in keeping with these findings because glucosamine, a component of neolacto GSLs, was absent from hydrolysates of neutral GSLs from poorly differentiated cells of two patients with M1 type of AML but was present in neutral GSL hydrolysates of M2-M4 AML cells. We found also some galactosamine in neutral GSLs of several of our patients but it could have been derived from contaminating lymphocytes. Leukemic leukocytes were also reported to be deficient in gangliosides (reviewed by Macher *et al.*, 1982). We have indeed observed a decrease of gangliosides in leukemic leukocytes of all our patients except patient Do. In relation to total carbohydrates, gangliosides were, however, deficient only in some patients.

We consider the results of this study as only preliminary. A decrease of protein-bound carbohydrates in immature white cells from patients with AML is a fact but caution should be exerted in extending this conclusion to other types of cells. For this purpose further studies are required both with leukemic leukocytes of different lineages and non-neoplastic cells maturing under normal conditions.

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