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Short Communication

QUANTITATIVE ANALYSIS OF LacCer/CDw17 IN HUMAN MYELOGENOUS LEUKAEMIC CELLS

JUSTYNA SPYCHALSKA¹, GABRIELA SMOLEŃSKA-SYM^{1*}, EWA
ZDEBSKA¹, JOLANTA WOŹNIAK² and JERZY KOŚCIELAK¹

¹Department of Biochemistry, and ²Department of Pathophysiology, Institute of
Haematology and Blood Transfusion, Chocimska 5, 00-957 Warsaw, Poland

Abstract: LacCer/CDw17 is the most abundant GSL in neutrophils. The cell-surface and intracellular presence of LacCer was determined quantitatively using anti-CDw17 mAbs in a flow cytometry assay. The quantified alterations in the level of CDw17 antigen expression are consistent with alterations in LacCer content, determined chemically. Our results show that CDw17 antigen expression defines successive stages in the maturation of the myeloid cell. The assessment of cell-surface and intracellular CDw17 expression may be useful in evaluating neutrophil physiological status.

Key Words: Neutrophils, Myelogenous Leukaemic Cells, Lactosylceramide, CDw17, Flow Cytometry

INTRODUCTION

LacCer/CDw17 (Gal β 1 \rightarrow 4Glc β 1 \rightarrow Cer) is abundant in biological material as a precursor of more complex GSLs. In recent years, research has shown that LacCer actively participates in such processes as the differentiation, growth and proliferation of cells, signal transduction, adhesion, and activation of phagocytes. Moreover, as a mitogenic factor, LacCer is involved in the pathogenesis of proliferative disease [1].

* Corresponding author: E-mail: gabsym@ihit.waw.pl

Abbreviations used: AML - acute myelogenous leukaemia; Cer - ceramide; CML - chronic myelogenous leukaemia; DAKO QIFIKIT - quantitative analysis of indirect immunofluorescence staining in flow cytometry; Gal - galactose; GalGalCer - galabiosylceramide; Glc - glucose; GSL - glycosphingolipid; HPTLC - high performance thin layer chromatography; LacCer - lactosylceramide; mAb - monoclonal antibody.

In the bone marrow, LacCer/CDw17 is a marker of myeloid line cells at the postproliferative stage [2]. In normal mature neutrophils, LacCer is a predominant GSL. It is mainly located inside the cells, where it is bound to granule membranes; the remaining amount (over 10%) is present on the cell surface membrane [3]. According to recent reports, the cell-surface LacCer forms LacCer-enriched GSL microdomains which mediate superoxide generation in neutrophils [4].

As shown previously [5, 6], at the various maturation stages of leukaemic cells, granule formation is accompanied by increases in LacCer levels. Therefore, LacCer may be a determinant of the maturity stage of myeloid cells, and it may also facilitate differential diagnoses of AML and CML.

Using an anti-CDw17 mAbs, we decided to test the usefulness of the DAKO QIFIKIT in the study of surface and intracellular expression of the LacCer antigen in normal and leukaemic myeloid lineage.

MATERIALS AND METHODS

Patients

Blood samples were collected from: 12 healthy donors; 16 patients with AML; and 4 patients with CML. The disease subtype was classified according to the French-American-British criteria.

Cell isolation and LacCer determination

Human peripheral blood neutrophils and leukaemic cells were isolated in a self-generated Percoll gradient via the method by Pertoft *et al.* [7]. The immunophenotype of the cells was defined by flow cytometry using the following mAbs: CD34, HLA-DR, CD71, CD45/CD14, CD13/CD33, CD15/CD117 and CD16/CD66.

LacCer from the isolated cells was extracted and purified, and the amount determined by the methods described previously [8]. Briefly, the lyophilised material was sequentially extracted with chloroform/methanol/water (1:1:0.2, v/v), chloroform/methanol (2:1, v/v), chloroform/methanol (1:2, v/v) and propanol/hexane/water (55:25:20, v/v). The extracts were combined and desalted using octadecyl Bakerbond spe columns. The total lipids in the dried extracts were acetylated, and chromatographed on Florisil columns (Bakerbond spe). Fractions containing GSLs were deacylated, and subsequently separated into individual compounds on silica gel 60 HPTLC plates (Merck) with the solvent system chloroform/methanol/water (65:35:8, v/v). GSLs were visualised with primulin spray, and spots containing diacylceramides (LacCer and GalGalCer) were scraped from the plates. These scrapings were hydrolysed with 2M trifluoroacetic acid for 4 h at 100°C. The hydrolysates were analysed for carbohydrates by High pH Anion Exchange Chromatography (Dionex Series 4500i system). The amount of LacCer was expressed in pmol of Glc/10⁶ cells.

Flow cytometric analysis

The expression strength of the GSL antigen LacCer/CDw17 in the whole blood cells was determined with the primary anti-CDw17 mAbs (IgM, clone MEM74, Serotec). Additionally, anti-CDw17 expression was evaluated in the isolated CML cells. The semiquantitative determination of the amount of the antigen was done using the DAKO QIFIKIT and TallyCAL for Windows computer software (DAKO). For intracytoplasmic LacCer analysis, a saponin-based permeabilisation reagent (IntraPrep, Immunotech) was used. The mAb-labelled cells were evaluated in a flow cytometer (CytronAbsolute, Ortho or FACSCalibur, Becton-Dickinson). 10000 cells were analysed each time. The results were presented as the amount of mAb/cell, which is proportional to the amount of antigen.

RESULTS

The total amount of LacCer in myeloid line leukaemic cells and normal mature neutrophils is listed in Tab. 1. The strength of the cell-surface and intracellular expression of the CDw17 antigen is shown in Fig. 1 (particular histograms are representative of each study type of leukaemia and control material).

Tab. 1. Altered LacCer content in myelogenous leukaemic cells and normal mature neutrophils.

		LacCer [pmol/10 ⁶ cells]			
		Type of leukemia			Normal cells
	AML		CML		
	M-2	M-3	myelo- /metamyelocytes	neutrophils	neutrophils
M-1	132.2	76.3	564.0	383.5	408.3
	± 76.2	± 19.4	± 50.2	± 46.3	± 30.3
	n = 4	n = 3	n = 4	n = 4	n = 12

Results are reported as the mean ± SD.

The least differentiated myeloblasts present in AML M1 are characterised by a low level of LacCer (23.9 pmol/10⁶ cells). The DAKO QIFIKIT test showed no cell-surface expression of CDw17, and its intracellular expression was low. In the more differentiated myeloblasts (AML M2), characterised by intense formation of primary granules, an increased LacCer synthesis was observed (5-fold greater than in M1). The presence of CDw17 was detected both on the cell surface (353 ± 244 mAb/cell) and intracellularly (5107 ± 2791 mAb/cell).

In leukaemic promyelocytes (AML M3), the LacCer level does not increase, and is even lower than in M2 cells (Tab. 1). The completed process of primary granule formation and incessant cell divisions at this stage of development may give rise to a decreased LacCer level. The above leads to granule dilution, and in view of this fact, the expression of CDw17 would not reflect the degree of cell maturity.

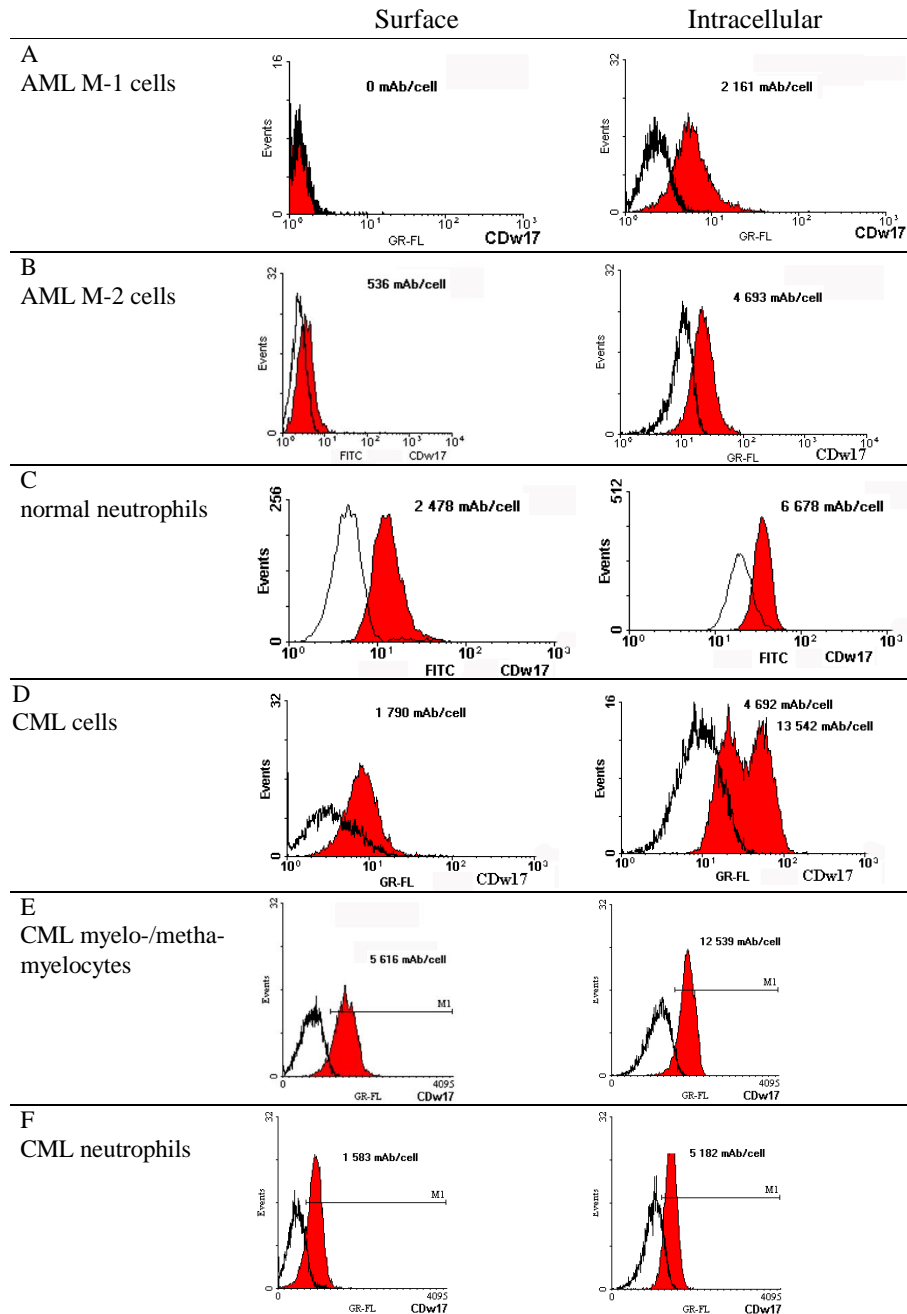


Fig. 1. Representative flow cytometry measurements of cell-surface and intracellular expression anti-CDw17 in myelogenous leukaemic cells and normal neutrophils. Control: irrelevant staining IgM ($\frac{3}{4}$). A-D: whole blood cells were subjected to staining, E, F: cells stained after isolation on a Percoll gradient.

The evaluation of CML cells gave a double-peaked histogram with different levels of cytoplasmic CDw17 expression when cell staining was performed on whole blood (Fig. 1D).

Therefore, the CML cells were fractionated by centrifugation in a density gradient into two fractions, of low and high density, containing a mixture of myelo-/metamyelocytes and mature neutrophils, respectively. Microscopic and flow cytometry examination showed the cells to be normal, both with respect to their morphology and expression of the CD16 and CD66 antigens.

The highest LacCer level (564.0 pmol/10⁶ cells) and the highest antigen CDw17 expression, both on the surface and inside the cells, were observed in the fraction of myelo-/metamyelocytes (CD16^{+/+}, CD66⁺) (Fig. 1E). These probably result from the formation of secondary granules at this stage of cell maturation. Fully mature neutrophils (the subfraction of CML neutrophils, CD16⁺⁺, CD66⁺) show a lower content of LacCer as compared to the fraction of myelo-/metamyelocytes. These differences were statistically significant at $p < 0.02$. The cell-surface and intracellular binding of the anti-CDw17 mAbs in the same cells was also reduced (Fig. 1F versus 1E).

In normal mature neutrophils, the LacCer content is similar to that found in CML neutrophils. As regards CDw17 expression, fully mature normal cells do not differ from leukaemic neutrophils. The intracellular expression of CDw17 is higher than its expression on the cell surface (5481 ± 1321 versus 1551 ± 517), which is consistent with the data reported in literature on LacCer localisation in normal neutrophils [9].

DISCUSSION

Neutrophils have an exceptionally high LacCer content (over 10-fold greater than the remaining blood cell types). LacCer molecules, like other GSLs, form clusters within cell membrane domains which are essentially insoluble in detergent, and are known as detergent-insoluble membranes or glycolipid-enriched membranes. In neutrophils, such domains are present in the plasma and granule membranes and contain the integral membrane protein, stomatin [10]. Iwabuchi and Nagaoka [4] provided evidence that LacCer microdomains are involved in the initiation of a signal transduction pathway leading to superoxide generation. This confirms previously reported data on the role of LacCer in the processes of neutrophil activation [9, 11, 1].

In view of the biological significance of LacCer, we decided to assess the expression of CDw17 in neutrophils and to simultaneously perform chemical evaluations of the compound. The use of DAKO QIFIKIT facilitated the semiquantitative analysis of the cell-surface and cytoplasmic antigen (after IntraPrep permeabilisation procedure). Our results showed that the amount of thus evaluated cell-surface- and intracellularly-bound anti-LacCer mAbs reflect the LacCer levels determined chemically. The test helps in the performance of an immediate quantification of the amount of LacCer (without the disruption caused by cell fractionation procedure) and in the avoidance of the tiring and

time-consuming procedure of cell separation, membrane isolation and lipid extraction.

The use of the anti-CDw17 mAbs also allowed us to evaluate the maturity status of the myeloid line leukaemic cells in the material obtained from patients with AML M1 and M2 and CML. An increased intracellular expression of the CDw17 antigen is correlated with the formation of primary granules at the myeloblast stage (M1, M2) and secondary granules at the myelo-/metamyelocyte stage (CML). At the same time, the cell-surface LacCer level increases (from 0 for M1 cells), reaching its peak at the myelo-/metamyelocyte stage. Previous reports merely described the cell-surface expression of LacCer on normal myeloid-line cells in the bone marrow [12, 2]. These data showed that the expression of CDw17 is dimly on immature cells, and increases significantly only at the metamyelocyte stage; this was confirmed by our studies of peripheral blood leukaemic cells.

It is worth emphasising that at the full maturity stage, both normal and CML neutrophils show a lower LacCer level and degree of CDw17 expression than less mature CML myelo-/metamyelocytes. This suggests that once neutrophils have entered the bloodstream, they may undergo many unknown processes accompanied by granule displacement, fusion with the plasma membrane, and degranulation. An opportunity to carry out a prompt assessment of the cell-surface and intracellular localisation of LacCer may help evaluate the physiological status of neutrophils.

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