Differentially regulated expression of 9-O-acetyl GD3 (CD60b) and 7-O-acetyl-GD3 (CD60c) during differentiation and maturation of human T and B lymphocytes

Dirk Wipfler1, G. Vinayaga Srinivasan2, Haneen Sadick4, Bernhard Kniep5, Sigrid Arming5, Martina Willhauck-Fleckenstein2, Reinhard Vlasak6, Roland Schauer3, and Reinhard Schwartz-Albiez1

GD3 (CD60a) and its 9-O-acetylated variant (CD60b) are intracellular regulators of apoptosis in T lymphocytes. Surface expressed 9-O-acetyl and 7-O-acetyl-GD3 (CD60b and CD60c) may have a functional impact on activated T and B cells. In order to investigate the balance between surface and intracellular expression and synthesis and degradation of these glycosphingolipids in human lymphocytes of various differentiation stages, we analyzed (i) expression of GD3 molecules on native T and B cells and thymocytes by flow cytometry and (ii) activity and regulation of possible key enzymes for CD60a,b,c synthesis and degradation at the transcriptional level. Both, surface and cytoplasmic expression of CD60a and CD60c was highest in tonsillar T cells. In thymocytes, CD60c outweighs the other CD60 variants and was mainly found in the cytoplasm. All lymphocyte preparations contained sialate O-acetyltransferase activity producing 7-O-acetyl-GD3. Sialidase activity was highest in peripheral blood lymphocytes followed by thymocytes and tonsillar T and B cells. Transcription of GD3 synthase (ST8Sia1), the key enzyme for GD3 synthesis, was highest in tonsillar T cells, whereas transcriptional levels of sialidase NEU3 and O-acetylerasere H-Lse were lowest in activated T cells. This balance between enzymes of sialic acid metabolism may explain the strong overall staining intensity for all GD3 forms in T cells. Both CASD1, presumably encoding a sialic acid-specific O-acetyltransferase, and H-Lse showed highest transcription in peripheral B lymphocytes corresponding to the low expression of CD60b and c in these cells. Our data point to regulatory functions of these anabolic and catabolic key enzymes for the expression of GD3 and its O-acetylated variants in lymphocytes at a given differentiation stage.

Keywords: CASD1 / CD60a,b,c / GD3 / lymphocytes / sialic acid O-acetylation

Introduction

Modification of sialic acids by O-acetylation has been recognized as a regulatory mechanism which plays a role in maturation and cellular differentiation processes and in fine-tuning of distinct receptors (Blum and Barnstable 1987; Schauer 2009). Although O-acetylation can occur at various sites of the sialic acid molecule, O-acetylation at carbon 7 and 9 is prevalent for this modification in lymphocytes. Further, O-acetylated variants of the ganglioside GD3, namely 7-O-GD3 and 9-O-GD3 have been described to be expressed on T and B lymphocytes in various intensities (Kniep et al. 1993; Fox et al. 2001). Several functions have been ascribed to GD3 and its O-acetylated derivatives. Intracellular GD3 is a messenger in the apoptotic pathway induced by the CD95 pathway (Malisan and Testi 2002), whereas 9-O-acetylated GD3 can suppress GD3-mediated pro-apoptotic effects (Chen and Varli 2002; Malisan et al. 2002; Kniep et al. 2006; Mukherjee et al. 2008). The function of GD3 and its O-acetylated variants on the lymphocyte cell surface is not yet clearly defined. Some observations point to an involvement of O-acetylated gangliosides in lymphocytic activation processes. Monoclonal antibodies against 9-O-acetylated GD3 (CD60b) can transfer costimulatory signals in T and B lymphocytes, whereas an antibody against 7-O-acetylated GD3 (CD60c) alone was sufficient to induce proliferation in these cell types (Welte et al. 1987; Fox et al. 1989; Claus et al. 1994; Erdmann et al. 2006). The differential agonistic effects of the antibodies may correlate to a different distribution of the respective gangliosides in the lymphocyte plasma membrane. 9-O-acetyl-GD3 was found enriched in raft-like cell surface domains, whereas 7-O-acetyl-GD3 showed a more homogeneous distribution on the cell surface (Erdmann et al. 2006).
Synthesis and expression of gangliosides and their derivatives depend on a timely and spatially balanced interplay of anabolic and catabolic enzymes (Gabius et al. 2002; Lauc et al. 2010; Willhauck-Fleckenstein et al. 2010). The key enzyme for synthesis of GD3 is the α2,8-sialyltransferase GD3 synthase (ST8SiaI). Several sialidases, which catalyze the removal of sialic acids from gangliosides, have been described for the human system and have been named NEU1, 2, 3 and 4, respectively (Monti et al. 2004, 2010). While NEU1 is a lysosomal enzyme degrading both sialoglycoproteins and glycolipids, NEU3 seems to be involved in the regulation of sialylation and thus modulation of the expression and function of cell surface gangliosides (Papini et al. 2004; Monti et al. 2010). A special feature of NEU3 is its presence in plasma membranes, suggesting that it may be involved in modulation of cell surface-expressed gangliosides. A preference of NEU3 expressed in the neuronal system and myelomonocytic cells within the hematopoietic cell system for GM3 has been described (Jin et al. 2008); however, depending on the cell type, other gangliosides may also be targets for this enzyme. Enzymes for the synthesis and degradation of O-acetylated gangliosides are still less well defined. Sialate O-acetyltansferase (SOAT) activities (EC2.3.1.45) were found in several species and organs (Higa et al. 1989; Shen et al. 2004; Lrho et al. 2007) and are mainly localized in the Golgi membranes. We have recently identified the human CASD1 (CAPsuLe Structure 1 Domain containing 1) gene encoding the Cas1 protein with an SGNH hydrolase and a hydrophobic transmembrane domain which may catalyze 7-O-acetylation of GD3 (Arming et al. 2011). NEU3 is able to desialylate 9-O-acetyl-GD3, although much slower than non-esterified GD3 (Oehler et al. 2002). It may be that in live cells the acetyl residue is removed by a sialic acid-specific O-acetylerase (SOAE; EC3.1.1.53) prior to degradation by sialidases. SOAE activity has been measured in various species of the mammalian system (Schauer and Shukla 2008; Srinivasan and Schauer 2008). In humans, H-Lse located in testis was described to encode a lysosomal SOAE (Zhu et al. 2004). The importance of degradation of O-acetylated sialic acids for immune regulation became recently apparent. It was found that a 9-O-acetyl sialic acid esterase is a negative regulator of the B lymphocyte antigen receptor (Cariappa et al. 2009) and mutations in the respective gene can be linked to human autoimmune disorders (Surolia et al. 2010).

In order to obtain a first insight into the regulation of O-acetylated GD3 in human lymphocytes, we measured transcription of GD3 synthase, CASD1, NEU1 and 3 and H-Lse in various T and B lymphocyte subsets representing various differentiation and activation stages. Transcription of these enzymes was compared with intracellular and cell surface expression of GD3 and its O-acetylated variants. In addition, the cellular SOAT and overall sialidase activities were estimated by enzymatic means.

Results

Comparison of surface and intracellular expression of GD3 and its O-acetylated variants at various stages of human lymphocyte differentiation

In order to assess the expression of CD60 antigens on the cell surface and within the cytoplasm, we stained T and B lymphocytes of various stages of differentiation with specific monoclonal antibodies against these gangliosides and simultaneously with either anti-CD3 or anti-CD19 antibody in order to discern T and B lymphocytes. In peripheral blood lymphocytes (PBLs), consisting in their majority of small resting lymphocytes, surface expression of CD60 was more pronounced in CD3+ T than in CD19+ B lymphocytes carrying CD60b and c. Interestingly, always two distinct populations of CD3+ T lymphocytes, namely a CD60 negative and a positive one were present (Figure 1). In contrast, CD19+ B lymphocytes were almost negative on their surface for CD60a and c and slightly positive for CD60b with no distinction between subpopulations. When cells were analyzed after permeabilization of the plasma membrane to allow intracellular antibody staining, in both cell types strong binding of antibodies against the three CD60 variants was visible, albeit in T lymphocytes to a larger intensity than in B lymphocytes (Figure 1). Intracellular staining was always strongest for CD60c. Although after permeabilization remaining surface staining cannot totally be excluded, we presume also because of the different staining pattern that the major part of stained antigens is located intracytoplasmically.

Tonsillar lymphocytes consist of lymphocytes in various stages of activation after contact with exogenous antigens. Here, surface expression of CD60 in CD3+ T cells was much stronger when compared with PBLs (Figure 1). A shift from the CD60 negative to the CD60 positive cell subpopulation, most distinctive for CD60c, was observed. CD19+ tonsillar B lymphocytes displayed a much weaker surface binding of CD60 antibodies. In CD3+ T lymphocytes, the intracellular pool of CD60 variants was enhanced when compared with CD3+ PBLs, whereas in CD19+ tonsillar B lymphocytes the intensity of staining remained similar, though the curves of CD60 positive cells were broader.

As a third group of lymphocytes, we analyzed thymocytes for their expression of CD60 antigens. T cell precursors undergo differentiation within the thymus to mature T lymphocytes. The entire population of thymocytes is positive for CD60a and CD60c on the cell surface and carries a larger intracellular pool of CD60c and less of CD60a and b (Figure 2). Stages of thymocyte differentiation can be distinguished by changes in surface markers, such as CD3, CD4 and CD8. Earliest stages are characterized by CD3-/CD4-/CD8-, then CD3-/CD4-/CD8- (“double negative”), followed by CD4+/CD8- (“double positive”) which then differentiate into either single-positive CD4+ or CD8+ thymocytes ready for export into the periphery. In our thymocyte preparations, the majority of cells consisted of double-positive thymocytes (Figure 2B, upper right quadrant), followed by CD4+ cells, double-negative thymocytes and CD8+ cells (Figure 2). When looking at CD60 expression in these thymocyte subpopulations, a characteristic feature is that CD4+ cells have the strongest and CD8+ cells the weakest surface expression of CD60b (Table 1). On the other hand, double-positive cells have the strongest surface expression of CD60a and CD60c. Finally, in all four subpopulations, the largest intracellular pool was observed with CD60c. This relative distribution of surface and intracellular CD60 antigens could be followed up in two independent thymocyte preparations.

A summary of surface and intracellular distribution of CD60 antigens in the three lymphocyte preparations is depicted in Figure 3.
Fig. 1. Flow cytometric analysis of CD60 variants in peripheral blood and tonsilar T and B lymphocytes. Percentage of positive cells (x-axis in logarithmic scale) is plotted against mean fluorescence intensity (y-axis). CD60a was stained by monoclonal antibody R24, CD60b by MT6004 and CD60c by U5. The grey shaded curves represent the respective isotype control stainings.
In a previous study, we observed that monoclonal antibodies against CD60b had a costimulatory and against CD60c a mitogenic effect both on human T and B lymphocytes. Further, in immunofluorescence microscopy CD60b binding appeared in the patchy, raft-like form, whereas CD60c displayed a more uniform distribution at the cell surface (Erdmann et al. 2006). To analyze the distribution of GD3 and its variants in raft-like (detergent-insoluble membrane, DIM) fractions, we isolated DIM and non-DIM fractions from membrane preparations of tonsillar T and B lymphocytes and measured gangliosides separated on subsequent thin-layer chromatography for their binding reactivity with the respective antibodies. In a first step, we tested DIM isolation on human lymphocytes and analyzed the expression of three different antigens of the MOLT-4 T lymphocyte cell line by western blotting (Figure 4A). Although major histocompatibility complex (MHC) class I was present in the DIM fraction, a stronger band was seen in the non-DIM fraction. In contrast, the leukocyte-specific protein tyrosine kinase (Lck), which assists in T cell signaling mediated by the T cell receptor, showed a stronger presence in the DIM fraction (Barbat et al. 2007). Cytoskeleton-associated phosphoprotein moesin (Schwartz-Albiez et al. 1995) was absent in the DIM fraction. Lck had been described as raft-associated molecule, whereas cytoskeleton proteins may only occur in rafts after activation of the cell (Sorice et al. 2004). Next, we used the same membrane fractionation procedure to isolate gangliosides from DIM and non-DIM fractions of T and B lymphocytes. The high-performance thin-layer chromatography (HPTLC)-separated fractions were subsequently stained with CD60b antibody MT6004 (Figure 4Ba). Since gangliosides after separation on HPTLC were incubated in an alkaline milieu to transform 7-O-acetyl-GD3 into 9-O-acetylated GD3, gangliosides at the position of 7-O-acetyl-GD3 could then also be stained by antibody MT6004. The presence of 9-O-acetyl-GD3 was clearly seen in the non-DIM fraction of T cells and to a smaller extent in the respective DIM fraction. In B cells, 9-O-acetyl-GD3 was present more strongly in the DIM fraction as deduced from the intensity of bands. With this method, 7-O-acetyl-GD3 could not be detected in human T and B lymphocytes. It may be that during the preparation of gangliosides, already a spontaneous shift from 7-O-acetyl to 9-O-acetylated GD3 occurred, as it had been described earlier (Kamerling et al. 1987; Vandamme-Feldhaus and Schauer 1998). In a second step, the HPTLC plate was additionally incubated with the anti-GD3 antibody R24. The non-DIM fraction of the T cell preparation seemed to contain more GD3 (Figure 4Bb, lower bands) than the DIM fraction. In contrast, in B cell preparations, DIM fractions

**Table I. Expression of CD60 variants on thymocyte subgroups**

<table>
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<tr>
<th>Antibody</th>
<th>CD4⁺/CD8⁺</th>
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<td>s</td>
<td>424⁺</td>
<td>515</td>
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<td>387</td>
</tr>
<tr>
<td>c</td>
<td>771</td>
<td>1839</td>
<td>766</td>
<td>733</td>
</tr>
<tr>
<td>CD60b (MT6004)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<tr>
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<td>981</td>
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<td>c</td>
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<td>7391</td>
<td>4202</td>
<td>5753</td>
</tr>
</tbody>
</table>

s, surface expression; c, cytoplasmic expression.

*Mean fluorescence values of flow cytometric analysis are given.*

**Fig. 2.** Expression of CD60 variants in human thymocytes (A) and distribution of CD4/CD8 in thymic subpopulations (B). (A) Thymocytes were stained with CD3 and with either R24 (CD60a), MT6004 (CD60b) or U5 (CD60c). Percentage of positive cells (x-axis in logarithmic scale) is plotted against mean fluorescence intensity (y-axis). The grey-shaded curves represent the respective isotype control stainings. (B) Two-dimensional plotted staining of CD3⁺ thymocytes with anti-CD4 and anti-CD8 monoclonal antibodies of one representative experiment. On x- and y-axes, the percentage of positive cells is indicated in logarithmic scales. Percentage of positive cells within each subgroup is given in numbers.

**Distribution of CD60 antigens in DIM fractions of T and B lymphocytes**

In a previous study, we observed that monoclonal antibodies against CD60b had a costimulatory and against CD60c a mitogenic effect both on human T and B lymphocytes. Further, ...
showed a stronger band of GD3 than non-DIM fractions. Double bands in the region of 9-O-acetyl-GD3 and GD3 may represent these glycosphingolipids expressed with a different fatty acid moiety.

Radioactive TLC analysis of lymphocytes SOAT assay
Analysis of the enzymatic reaction product on silica gel TLC showed that the migratory position of the neo-O-acetylated ganglioside corresponds with 7-O-acetylated GD3 standard (Figure 5). There was only little activity at the position of 9-O-acetyl-GD3. 7-O-acetylated GD3 was the primary reaction product of SOAT from all lymphocytes tested. Lymphocyte SOAT was inactive with CMP-Neu5Ac. Table II indicates the SOAT activities determined with GD3 as a substrate in various lymphocyte subsets. The highest activity was observed in thymocytes, whereas the lowest activity was seen in peripheral blood T cells. Peripheral B cells showed slightly higher activity when compared with T cells, whereas the tonsillar T and B cells exhibited similar activities.

On incubation of the reaction product with sialate-9-O-acetylesterase, no reduction in the radioactive peak of the neo-O-acetylated ganglioside occurred, which indicates that this is not a 9-O-acetylated GD3 product but rather 7-O-acetylated GD3 according to the cochromatography with 7-O-acetylated GD3.

Analysis of lymphocyte sialidase activity
For the determination of overall sialidase activity in various lymphocyte subsets, we used 4-MU-NeuAc as a substrate in a fluorimetric assay as described (Zanchetti et al. 2007). PBLs followed by thymocytes had the highest values of sialidase activity, whereas tonsillar T and B cells showed sialidase activity at comparatively low levels (Table III).

Analysis of enzymes involved in synthesis and degradation of GD3 and its O-acetylated variants at the transcription level
The key enzyme for the synthesis of GD3 is GD3 synthase (ST8SiaI). Several sialidases are known which are able to hydrolyze sialic acid from gangliosides (Monti et al. 2010). NEU3 present in endosomal compartments and on the cell surface degrades α2,3- and α2,8-linked sialic acids from gangliosides and also degrades O-acetylated sialic acids albeit much slower (Schauer and Shukla 2008). NEU1 is a lysosomal sialidase. We could not detect NEU2 and 4 in human lymphocyte subsets here (data not shown). In contrast, enzymes regulating synthesis and degradation of O-acetylated gangliosides are not yet well characterized. CASD1 presumably represents an evolutionary conserved gene encoding for a sialic acid-specific O-acetyltansferase in humans for which specificity for gangliosides was not known previously. We could show that after transfection of the CASD1 cDNA alone or co-transfected with ST8SiaI cDNA into COS-7 cells expression of 7-O-acetyl-GD3 increased (Arming et al. 2011). A sialic acid-specific lysosomal O-acetyletlase (H-Lse) was first characterized in mice and its homolog identified in human testis (Zhu et al. 2004).

Fig. 3. Schematic overview of CD60 variant distribution between surface and intracellular compartments in various human lymphocyte subsets. For details, see text.
feature was that tonsillar lymphocytes had always the lowest levels of NEU1, NEU3 and H-Lse.

Discussion

GD3 and its O-acetylated variants have been shown to be involved as cell surface structures in lymphocyte activation and as intracellular substances in the regulation of apoptosis (Giammarioli et al. 2001; Malisan and Testi 2002; Garafalo et al. 2007; Malorni et al. 2007). Much effort has been undertaken to elucidate the pro- and anti-apoptotic mechanisms mediated by GD3 and 9-O-acetyl-GD3, respectively. For instance, it was demonstrated that mitochondrial reactive oxygen species oxidative processes may transform GD3 into GD3-7-aldehyde, which has a higher proapoptotic activity than GD3 itself and further that 9-O-acetylation may block the oxidative modification of the sialic acid moiety of GD3 (Brenner et al. 2010). Although we have come closer to an explanation of intracellular GD3 functions at the molecular level, many questions are still waiting for resolution. For instance, what regulates (i) the intracellular transformation of GD3 into the 7-O-acetyl derivative and further into 9-O-acetyl-GD3 and (ii) subsequently the differential transport of GD3 and its O-acetylated forms to the cell surface?

In a first attempt, we tried to assess the balance between intracytoplasmic content and surface expression of GD3 and

Fig. 4. Characterization of human lymphocyte DIM fractions. (A) DIM (a) and non-DIM (b) fractions were prepared from MOLT-4 T cells as described in Materials and methods, western-blotted and stained with monoclonal antibodies against MHC class I, Lck and moesin. (B) Gangliosides were isolated from DIM and non-DIM fractions of tonsillar T and B lymphocytes and stained with anti CD60b-specific monoclonal antibody MT6004 (a; overnight at 4°C). Note that gangliosides migrating to the position of 7-O-acetyl-GD3 could be detected by CD60b-specific MT6004 after alkali-induced shift of the O-acetyl group from C7 to C9. Lane 1, non-DIM of T cells; lane 2, DIM of T cells; lane 3, non-DIM of B cells; lane 4, DIM of B cells; lane 5, total T and B ganglioside fractions; lane 6, 0.1 µg of purified 9-O-acetyl-GD3; lane 7, 0.3 µg of purified 9-O-acetyl-GD3; lane 8, 0.1 µg of purified 7-O-acetyl-GD3 (after mild alkaline treatment). (b) Restaining of ganglioside fractions with monoclonal antibody R24 (2 h at room temperature) after washing [2 × with phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature and 1× with PBS containing 1 % (v/v) bovine serum albumin for 30 min at room temperature]. The sequence of lanes is identical to (a) after washing and staining with R24 (CD60b). The now appearing lower bands represent GD3, please note that staining with MT6004 will remain visible after restaining with R24 and that we used here a solvent for thin-layer chromatography which does not allow separation of GD3 and 7-O-acetyl-GD3.

Fig. 5. Radio thin layer analysis of lymphocyte SOAT assay. The SOAT assay reaction products after incubation of lymphocyte homogenates with GD3 and [14C]-AcCoA were co-chromatographed with 7-O-acetylated GD3 standard in HPTLC. Gangliosides were separated on HPTLC using chloroform/methanol/water containing 25 mM CaCl₂, followed by radioscanning and orcinol/ferric chloride staining. In the graph, the scanned migration of the neo-acetylated radioactive gangliosides together with the non-radioactive O-acetylated GD3 standards is shown. Below, the gangliosides stained for sialic acids with orcinol reagent are shown in the same migration measure to enable identification of the O-acetylated GD3 formed. The radioactive AcCoA peak is derived from the acetyl donor substrate. The enlarged chromatogram is also shown at the right side.
7-O-acetyl- and 9-O-acetylated GD3 as identified by the respective CD60a,b,c monoclonal antibodies in flow cytometry in human lymphocytes of various differentiation and activation stages. One has to note that this method provides an estimation of changes in these gangliosides rather than information on quantities of gangliosides in the given compartment. It became obvious that small resting CD3+ T lymphocytes derived from peripheral blood had a higher content of CD60 molecules than CD19+ peripheral blood B lymphocytes. This difference was also reflected by CD60 surface expression in both cell types. Interestingly, resting T lymphocytes displayed at their surfaces two populations with regard to CD60 expression, a negative and a positive one, while the intracytoplasmic pool of CD60 showed a homogeneous staining pattern. This was clearly not observed in B lymphocytes.

In vivo preactivated T lymphocytes, represented by tonsillar T lymphocytes, a shift in surface expression to the CD60+ subpopulation was observed, most distinct with regard to CD60c staining. In general, activation results in an increase in CD60 expression both in T and B lymphocytes as reported previously (Erdmann et al. 2006). The pool of intracytoplasmic CD60 is also increased in activated cells. In more detail, a considerable portion of GD3 seems to be transformed to 7-O-acetyl-GD3 and further to 9-O-acetyl-GD3. In contrast to the differential cell surface expression of CD60 in T cell subpopulations, no such distinction can be made by their intracytoplasmic pool of CD60. It therefore seems that there exists a mechanism not yet identified which regulates the surface expression of CD60 molecules during activation. In thymocytes, the pool of 7-O-acetyl-GD3 seems to be much larger than that of the precursor GD3. During the differentiation of thymocytes, the stage of CD4+/CD8+ cells displays the strongest surface and cytoplasmic staining for CD60a and c. With regard to CD60b expression, CD4+/CD8- thymocytes prevail over earlier thymocyte differentiation stages and also CD4+ /CD8+ T cells.

Gangliosides have a regulatory role in raft-like structures (Odintsova et al. 2006). Isolation of DIM and non-DIM fractions, subsequent separation of gangliosides and staining with CD60b antibody MT6004 and CD60a antibody R24 yielded an unexpected result considering the higher content of GD3 and its variants in T lymphocytes as measured by flow cytometry. DIM fractions of tonsillar B lymphocytes apparently contained more of 9-O-acetyl-GD3 and GD3 than the corresponding DIM fractions of T tonsillar lymphocytes, whereas in the non-DIM fraction of T lymphocytes, a larger quantity of these gangliosides was observed. In an earlier study, we found strong CD60c staining of raft-like surface structures on live tonsillar B lymphocytes as determined by fluorescence microscopy using the same monoclonal antibodies (Erdmann et al. 2006). In contrast, in isolated DIM and non-DIM fractions, CD60c could not be observed. It is most likely that during the preparation of gangliosides for DIM analysis, a spontaneous shift from 7-O-acetyl- to 9-O-acetyl-GD3 occurred so that staining with the CD60c antibody was negative (Kamerling et al. 1987; Vandamme and Schauer 1998). On the other side, in the sterical context of an intact cell surface, the 7-O-acetyl-GD3 is stabilized and even at treatment of live cells with culture medium of pH 9.0 for 10 min cell surface CD60c remained stably expressed (Erdmann et al. 2006).

SOAT activity has been measured in several species and cell systems (Butor et al. 1993; Mandal et al. 2009; Schauer 2009). We analyzed SOAT enzymatic activity in the T and B lymphocyte subpopulations. The radioactively labeled products were separated on TLC and the radioactivity incorporated quantitatively assessed. As product of the cellular SOAT activity in these human lymphocyte populations, only 7-O-acetyl-GD3 was identified. From these results, it seems that a specific 9-O-acetyl SOAT is not present in human lymphocytes. In further conclusion, it may be that 9-O-acetyl-GD3 is either a product of spontaneous or enzyme-mediated (hypothetical "migrase") O-acetyl migration from C7 to C9 as postulated earlier (Kamerling et al. 1987; Vandamme-Feldhaus and Schauer 1998). However, due to the distinct function of 9-O-acetyl-GD3 during apoptosis, we think that a coordinated translocation process is more likely than a spontaneous one. In our experiments, we could not detect large differences in SOAT activity between the lymphocyte populations investigated with the exception of an enhanced activity in thymocytes. Our results corroborate the sequence of events leading to O-acetylated GD3, namely that GD3 synthesis is followed by 7-O-acetylation and at a later stage by 9-O-acetylation.

### Table II. SOAT activity in various lymphocyte subsets

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<th>Enzymatic activity (pmol × mg⁻¹ × min⁻¹)</th>
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<td></td>
<td>B</td>
<td>504</td>
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<tr>
<td>Thymus</td>
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</table>

*T and B lymphocytes of peripheral blood and tonsils as well as thymocytes were separated as described in Materials and Methods, and homogenates of these cell preparations were tested for their SOAT activity in the radio-TLC assays as described in Materials and Methods and shown in Figure 5. The calculation of specific enzyme activity is based on the radioactive amount (cpm) of the neo-O-acetyl reaction product from radio TLC. The specific activity of radioactive AcCoA was used for calculation; shown are mean values obtained from three independent experiments. We always obtained similar results.\n
### Table III. Sialidase activity in various lymphocyte subsets

<table>
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<td>Thymus</td>
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Sialidase activity as measured with the 4-MU-NeuAc fluorometric assay was highest in peripheral lymphocytes followed by that of thymocytes and at a lower level of tonsillar T and B cells. Although this assay does not allow to differentiate between NEU1 and NEU3 activity, the enzymatic values correspond to the transcriptional activity measured. NEU1 transcription was also highest in peripheral lymphocytes. From this, we tentatively conclude that most of the sialidase activity determined originates from NEU1.

In order to get a closer view on the regulation of GD3 gangliosides, we measured the transcription of key enzymes of this pathway in real-time polymerase chain reaction (PCR) and additionally of those genes which encode glycan-degrading enzymes possibly also involved in GD3 metabolism. As expected and in accordance with surface and cytoplasmic staining results, transcription of GD3 synthase was much higher in T than in B cells. CASD1, which is most likely responsible for 7-O-acetylation in human cells (Arming et al. 2011), surprisingly was found with highest values in peripheral B lymphocytes and with comparatively lower values in activated tonsillar T lymphocytes. However, the overall situation is more complex and may explain these observations when looking at degrading enzymes. Sialidases like the lysosomal NEU1 and even more pronounced NEU3, which is also described to be expressed at the cell surface, are transcribed at low levels in tonsillar T lymphocytes. In a similar way, the lysosomal SOAE H-Lse is transcribed at much lower levels in T lymphocytes when compared with B lymphocytes. High levels of GD3 synthase and low levels of NEU1 and H-Lse transcription together may explain the higher expression both of GD3 and O-acetylated GD3 in this T cell fraction. In B cells, the situation appears in the opposite way: low transcription level of GD3 synthase and high transcription of NEU1 and H-Lse may result in a much lower expression of GD3 gangliosides when compared with T lymphocytes. The putative role of SOAE in murine B lymphocytes with regard to masking and unmasking of the B cell coreceptor CD22, and its subsequent consequences for B cell activation was discussed (Cariappa et al. 2009). One may further speculate that the balance between SOAT (possibly CASD1) and SOAE (H-Lse) decides to which extent lectin binding to unmasked sialic acid residues like CD22 (Siglec 2) can exert their functions. In this respect, it may be of interest that both the SOAE and the SOAT seem to regulate the expression of O-acetylated sialic acids in human colon mucosa (Shen et al. 2004).

Fig. 6. Schematic overview of transcriptional levels of enzymes investigated. Values [x-fold expression of lowest (=1) value shown] of real-time PCR for the genes encoding ST8Sia1, NEU1, NEU3, CASD1 and H-Lse derived from preparations of different human lymphocyte subsets are depicted.
In this study, we attempted to compare the overall expression of GD3 variants in live lymphocytes with the transcription of anabolic and catabolic sialic acid enzymes. The results obtained contribute to our understanding of a very complex regulatory mechanism leading to the synthesis and cellular expression of gangliosides and in particular their O-acetylated variants in lymphocytes. This study may stimulate further experiments regarding the expression and properties of the enzymes involved, such as the specificity of the esterase.

Materials and methods

For the analysis of GD3 and its O-acetylated variants, we used the monoclonal antibodies R24 (anti-GD3, CD60a) and U5 (7-O-acetylated GD3, CD60c; Kniep et al. 1995), kindly provided by Dr C. Claus, Mainz, and MT6004 (9-O-acetylated GD3, CD60b), kindly provided by Dr Rieber, Dresden. Anti-MHC class I antibody W6/32 was a gift from Dr Moldenhauer, Heidelberg, anti-LCK (Becton Dickinson, Heidelberg, Germany) and anti-moesin (38/87) was produced by one of the authors (RS-A; Schwartz-Albiez et al. 1995). As isotype controls, an IgM (G155-228) and an IgG3 (J606) preparation, both of Becton Dickinson, were applied. For subtyping lymphocytes, the following conjugated antibodies were applied: CD3-fluorescein isothiocyanate (FITC), CD3-PE, CD3-Cy-chrome, CD4-PE, CD8-Cy-chrome, CD14-PE, CD19-PE, CD19-PerCP-cy5.5 (all Becton Dickinson) and anti-IgM-isotype-FITC (Chemicon, Schwalbach, Germany). As secondary antibodies, we used goat anti-mouse IgG/IgM FITC and goat anti-mouse-AP (all Dianova, Hamburg, Germany).

We here use the CD60a,b,c nomenclature (Schwartz-Albiez 2002) throughout this study when describing antibody reactivities with GD3 and its variants.

Isolation of lymphocyte subtypes and in vitro cell culture

Normal PBLs were obtained from healthy donors and separated by centrifugation on Ficoll-Paque (Amersham, Freiburg, Germany). Tonsils were obtained from children undergoing tonsillectomy because of hyperplastic tonsillitis and were separated as described elsewhere (Bergler et al. 1999). Thymocytes of thymic tissue derived from young patients undergoing heart surgery were prepared as described (Gotter et al. 2004). When necessary lymphocytes were further separated into B and T lymphocytes by incubation with CD19− or CD3-coupled Dynabeads (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol.

Informed consent for the use of biopsy material was obtained from the patients and the parents of children patients in accordance with the Helsinki protocol.

Flow cytometric analysis

Multicolor flow cytometric analysis of cell surface antigens was performed as described (Erdmann et al. 2006) using a FACS Canto II and the FACSDIVA software (Becton Dickenson). Dead cells were excluded by staining with Via-Probe (Becton Dickenson) shortly before analysis. For staining of cytoplasmic antigens, plasma membranes were permeabilized and fixed by pre-suspending cells in Cytofix/Cytoperm (0.1% saponin, 4% formaldehyde; Becton Dickenson) solution for 20 min on ice and washed thereafter three times with Perm/Wash solution [0.09% NaN3, 1% (w/v) saponin]. For washing of cells between staining steps, Perm/Wash buffer was used. Due to detergent treatment, dead cells could not be excluded by Via-Probe staining before intracellular staining procedure. However, for intracytoplasmic staining experiments, only cell cultures were taken with less than 5% dead cells as measured by trypan blue exclusion.

Relative fluorescence intensity was calculated as: mean fluorescence intensity of sample/mean fluorescence intensity of isotype control × 100. In some experiments, percentage values for positive cells are given. Samples were assessed as positive when values were higher than those of isotype controls.

Isolation of membrane microdomains, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

Isolation of detergent-resistant membrane domains (DIM) of T or B lymphocytes separated by magnetic bead technology was essentially performed as described (Krämer et al. 1999). Cells were incubated in lysis buffer [10 mM Tris–HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% (w/v) of Triton X-100 and protease inhibitors (complete mini Roche, Mannheim, Germany), pH 7.5] for 20 min on ice, centrifuged for 5 min at 1300 × g and the supernatant mixed with a 85% (w/v) solution of sucrose in lysis buffer. The cell suspension was then added to ultracentrifugation vials, layers of 35% and finally 5% sucrose were subsequently overlaid. Centrifugation was performed in a SW55 rotor in a L8-70 Beckman ultracentrifuge at 200,000 × g for 18 h at 4°C without brake. The ring consisting of DIM components was carefully separated from the sucrose gradient, the next 500 µL were discarded and 1.5 mL of the lower density layer were used as “non-DIM” fraction.

Samples derived from sucrose density gradients were mixed with sodium dodecyl sulfate–sample buffer and incubated for 5 min at 95°C before loading on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins separated by SDS–PAGE were transferred to PVDF membranes and subjected to western blotting. After incubation of western blots with the respective primary and secondary antibodies, stained protein bands were visualized by the ECL technique.

Isolation of gangliosides and HPTLC

Cellular samples were resuspended in chloroform–methanol (2:1, v/v), and the extracted gangliosides were purified and subsequently separated on HPTLC as described elsewhere (Brenner et al. 2010). TLC immunostaining of defined glycosphingolipid standards and gangliosides separated from cellular extracts was performed as described (Kniep et al. 1993).

RNA isolation, cDNA synthesis and real-time PCR

Cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the
manufacturer’s instruction. RNA was eluted in water. The quality of total RNA was checked by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values >8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

About 1.0 µg of total RNA was Oligo(dT) primed and first-strand cDNA synthesis was performed according to the manufacturer’s guidelines (Super Script™ First-Strand Synthesis System for RT–PCR, Invitrogen).

For quantification of mRNA expression, cDNA samples were analyzed by real-time quantitative PCR. A total of 125 ng of cDNA was amplified in 25 µL using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the presence of 900 nmol of the specific primers (Table IV) using the 7300 Real Time PCR system (Applied Biosystems, Darmstadt, Germany). Oligonucleotide specificity, synthesized by MWG Biotech (Ebersberg, Germany) was computer-tested (BLAST, NCBI) by homology search with the human genome; primers used are listed in Table IV. Samples were run in triplicate and experiments were repeated twice. The thermal profile for the reaction was 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. In order to exclude non-specific amplification, dissociation curve analysis and agarose gel electrophoresis of the PCR products were performed at the end of the run. The endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase was chosen for normalization. Relative gene expression was calculated using the comparative Ct method (Livak and Schmittgen 2001).

Preparation of cell lysates from native lymphocytes for enzymatic analysis
Native lymphocytes (tonsillar T and B cells, peripheral T and B cells and thymocytes) were shock-frozen in liquid nitrogen for 30 s, followed by thawing at 30°C for 6 min with gentle mixing. The procedure was repeated four times and the cell lysate obtained was centrifuged at 6000 × g for 30 min at 4°C to pellet the cell debris. The supernatant containing microsomes was used for testing SOAT activity. Protein was quantified using the BCA assay kit according to the manufacturer’s (Interchim, Montlocon, France) instructions.

Sialate-O-acetyltransferase assay with GD3
In order to test enzymatic activity, we used GD3 gangliosides as a substrate according to the procedure described (Srinivasan and Schauer 2008). GD3 (25 µg; from SnowBrand Milk Products, Tokyo, Japan) was sonicated for 10 s in 50 mM phosphate buffer, containing 50 mM KCl, pH 6.5 (SOAT buffer), followed by the addition of 25 µg lymphocyte homogenate protein and 0.04 µCi [14C] AcCoA (specific activity 59 mCi/mmol; Amersham, Braunschweig, Germany) as a cosubstrate to yield 200 µL of assay volume. Incubation was carried out at 37°C for 30 min and the reaction stopped by shock-freezing in liquid nitrogen. The assay mixture was centrifuged at 14,000 × g for 30 min at 4°C in order to remove precipitates, concentrated to dryness by Speed Vac centrifugation, dissolved in 50 µL water and directly analyzed by radio-TLC.

About 20 µL were cochromatographed on silica gel with authentic 9- and 7-O-acetylated GD3 standards (produced by one of the authors, respectively, by BK). The plate was developed using chloroform:methanol:water containing 25 mM CaCl2 (5:4:1), followed by radio-TLC scanning using the LB 284 Berthold linear analyzer (Berthold, Wildbad, Germany) and orcinol/ferric chloride staining.

Sialate-9-O-acetylesterase treatment of neo-O-acetylated GD3
In order to get information regarding the position of the sialic acid O-acetyl group formed, an aliquot of the enzyme assay preparation was incubated with recombinant influenza C virus esterase (2.5 mU; produced by one of the authors, RV; Strasser et al. 2004) at 37°C for 15 min in the SOAT buffer (Srinivasan and Schauer 2008). This esterase can act only on 9-O-acetyl groups (Rogers et al. 1986). The reaction was terminated by shock-freezing. The supernatant obtained after centrifugation was analyzed by radioactive TLC on silica gel in Sialate-O-acetyltransferase assay with GD3.

Sialidase assay on lymphocyte preparations
Lymphocyte subsets were separated and cell lysates were prepared as in preparation of cell lysates from native lymphocytes for enzymatic analysis. The fluorimetric sialidase assay using 4-MU-NeuAc (4-methylumbelliferone neuraminic acid) as a substrate was performed essentially as described (Zanchetti et al. 2007). The reaction mixture (total volume:100 µL) was set up with an appropriate concentration of 4-MU-NeuAc in the presence of bovine serum albumin (40 mg/mL) and the sample protein preparations in various concentrations and was incubated at pH 3.8 for 30 min at 37°C. The reaction was stopped by addition of 150 µL of 0.2 M glycine/NaOH, pH 10.2. Fluorescence emission was measured on a Fluoroskan Ascent FL (Thermo Scientific, Dreieich, Germany) with excitation at 360 nm and emission at 450 nm, using 4-MU in various concentrations to set up a calibration curve.

### Table IV. Primers used for RT–PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD3-synthese (ST8Sia1)</td>
<td>F 5′-AGCGTTCAGGAAACAAATGG-3′  R 5′-TCACCTATTGACGCCACA-3′</td>
</tr>
<tr>
<td>O-acetyltransferase (CASD1)</td>
<td>F 5′-GTGGATTTCTGTGACATCC-3′  R 5′-AAGCCTTTCACTGCTACAT-3′</td>
</tr>
<tr>
<td>9-O-acetylesterase (H-Lse)</td>
<td>F 5′-CTACTGCCCCACCCCAAGAA-3′  R 5′-TTTAATCGGGTGAGTGGC-3′</td>
</tr>
<tr>
<td>Neuraminidase 1 (NEU1)</td>
<td>F 5′-TCGATGATTGAGCGCCTC-3′  R 5′-TACGCGGGCCTAGGTGATC-3′</td>
</tr>
<tr>
<td>Neuraminidase 3 (NEU3)</td>
<td>F 5′-TGCGAGGAGGCTTTCTACG-3′  R 5′-TCTGGCCCTGACACAACTCG-3′</td>
</tr>
<tr>
<td>β-Actin (ACTB)</td>
<td>F 5′-GCTCTCTGTGGGAGGCAGG-3′  R 5′-CACTGCTGAGGATGGGACA-3′</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
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Conflict of interest

None declared.

Abbreviations

CASD1, Capsule Structure 1 Domain containing 1; DIM, detergent-insoluble membrane; FITC, fluorescein isothiocyanate; H-Lse, sialic acid-specific lysosomal O-acetylated; HPTLC, high-performance thin-layer chromatography; Lck, leukocyte-specific protein tyrosine kinase; MHC, major histocompatibility complex; 4-MU-NeuAc, 4-methylumbelliferone; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOA, sialic acid specific O-acetylated; SOAT, sialate O-acetyltransferase; ST8Sial, α2,8-sialyltransferase; GD3 synthase.

References


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