Ecto-Sialyltransferase of Human B Lymphocytes Reconstitutes Differentiation Markers in the Presence of Exogenous CMP-N-Acetyl-Neuraminic Acid

By Hans-Jürgen Gross, Anette Merling, Gerhard Moldenhauer, and Reinhard Schwartz-Albiez

The existence of an ecto-sialyltransferase (ecto-ST) on B lymphocytes with increasing activity at late maturation stages is shown using a novel flow cytometric enzyme assay. This ecto-ST is effective in reconstituting different surface glycoconjugates on desialylated B cells in the presence of exogenous CMP-NeuAc. We found that this ecto-ST is distinct in its activity from soluble ST released into the culture supernatant. Surface sialylation was independent of the amount of ST secreted into the culture supernatant and followed different kinetics than sialylation of exogenous substrate by soluble ST. Four human B-cell lines representing different maturation stages were analyzed for secreted and ecto-ST activity. The myeloma cell line U266 and the lymphoblastoid cell line JOK-1 showed higher activity of both ST forms than the acute lymphoblastic leukemia B-cell line Nalm-6. ST activity in culture supernatants of U266, JOK-1, and Nalm-6 cells consisted predominantly of the α2,6 ST type with specificity for N-linked oligosaccharides. As an exception, the myeloma cell line IM-9, deficient of α2,6 ST activity, secreted only small amounts of ST and showed low activity of ecto-ST. Sialylation of surface-expressed glycoconjugates by ecto-ST was measured by incubating B-cell lines in the presence of fluorescent CMP-sialic acid. Surface structures labeled with fluorescent sialic acid under this condition were visualized by confocal laser scanning microscopy and fluorescent label was quantitatively assessed by flow cytometric analysis on live cells. Incubation of cells in acidified culture medium, to release possibly receptor-bound ST, did not alter the intensity of cell surface sialylation. Inhibition of internalization and membrane traffic by various approaches (reduced incubation temperature and chloroquine or brefeldin A treatment) did not block surface sialylation. Together, these observations point to cell surface sialylation in B lymphocytes mediated by a cell surface-expressed ecto-ST distinct from the secreted ST form. On desialylated JOK-1 cells, ecto-ST in the presence of exogenous CMP-NeuAc was able to resialylate the B-cell surface sialoglycans CD75 and HB-6 and major surface glycoproteins of B cells, such as HLA class I and II antigens, transferrin receptor, and surface IgM. In contrast, cell surface glycans of coincubated desialylated erythrocytes were not sialylated by the B-cell ecto-ST. Ecto-α2,6 ST of B cells may be involved in the sialylation of distinct differentiation glycan antigens.

Glycosyltransferases are integral membrane proteins of the Golgi apparatus.18 Also, soluble forms of glycosyltransferases have been identified in several body fluids.20 For instance, increased ST activity has been reported in sera of patients during inflammation and tumor growth.19,21 These soluble enzymes seem to originate from membrane-bound forms released by proteolytic cleavage of the NH2-terminal signal anchor.22 In earlier studies, murine B lymphoma,23 melanoma cells,24 rat hepatoma cells, and chicken neurons25 have been reported to produce extravascular ST activity that is able to sialylate cell surface structures. The presence of ST at the plasma membrane has been indicated by immunoelectron microscopy studies.26,27 The question whether ST exist as cell surface-associated molecule, possibly derived from secreted material, or as a distinct membrane-integrated enzyme was still a matter of debate.

Evidence has been provided for the existence of a cell
surface, membrane-integrated ecto-β1,4 galactosyltransferases. For the surface-expressed galactosyltransferase, a function in sperm-egg binding has recently been shown suggesting that glycosyltransferases themselves have the potential to act as adhesion molecules. Furthermore, cell surface β1,4 galactosyltransferase also seems to be catalytically active in that the enzyme transfers galactose residues to extracellular laminin.

Because cell surface-located (ecto-) ST may be involved in coordinated expression of functionally relevant sialoglycans on B lymphocytes, we addressed in this study the following questions. (1) Is ST activity measurable at the cell surface of B cells, possibly modulated in its intensity during B-cell ontogeny? (2) Is surface sialylation mediated by a membrane-located form of ST? (3) Is ecto-ST able to resialylate defined membrane-expressed sialoglycans, known as differentiation markers and as ligands for adhesion proteins? (4) Is cell surface ST catalytically active on cells being in close contact with the respective B cell? In an attempt to answer these questions, we analyzed ecto-ST activity of four differently matured human B-cell lines using a new, sensitive fluorometric ST assay. This assay allows to quantitatively determine sialylation of cell surface structures in flow cytometric analysis by use of synthetic fluorescent CMP-sialic acids. We show that B lymphocytes carry an α2,6 ST on their cell surface that may have a task in modulating certain surface carbohydrate structures.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany). Nylon membranes (Immobilon-P) were from Millipore (Bedford, MA). Cytidine 5′-triphosphate (CTP) was obtained from Biomol (Ilvela, Barcelona, Spain). Desialylation of glycoproteins was performed by acid hydrolysis as described. All cell lines were maintained in RPMI 1640 supplemented with 5% fetal calf serum. For determination of endogenous, ecto-ST activities, cells were cultivated for 24 hours in serum free FFHM II medium (GIBCO, Eggenstein, Germany) before the tests.

Cell line Nalm-6 carries markers of early B-cell maturation (CD10, CD19, CD24, and CD72). JOK-1 cells express markers of activated B cells (CD21, CD22, and CD75). Cell line U266 is deficient for almost all known B-cell differentiation markers except for CD44, CD73, and CD75, which characterizes late stages of B-cell activation. Similarly, cell line IM-9 expresses only few B-cell differentiation markers with either a broad presence during maturation reaching to the blast stage (CD39 and CD78) or with pronounced presence on activated B cells (CD23).

Antibodies. The CDw76 MoAb HD66 was produced in our laboratory. MoAb CRIS 4 of CDw76 was kindly donated by Dr. R. Villela (Barcelona, Spain). The CD75 MoAb HH2 was a generous gift of Dr. S. Funderud (The Norwegian Radium Hospital, Oslo, Norway). Antibodies HB-4 and HB-6 and MoAb VIB-E3 of the CD24 panel were obtained from the Fourth International Workshop On Human Leucocyte Differentiation Antigens. The MoAb 1B2 was produced using the respective hybridoma 1B2-1B7 obtained from the American Tissue Culture Collection (Rockville, MD). All MoAbs against carbohydrates were used here are of IgM isotype.

For immunoprecipitation studies, we used MoAb W6/32 against the human HLA class 1 molecule, MoAb L243 against HLA class II, MoAb PA-1 against the transferrin receptor (CD71), and MoAb M288.5 against the μ chain. A mouse anti-fluorescein isothiocyanate (FITC) MoAb was purchased from Serotec (Wiesbaden, Germany).

The following second immune reagents were used: goat antimouse IgG + IgM (Fab), conjugated to FITC (Dianova, Hamburg, Germany) and sheep anti-FITC (Fab) coupled to alkaline phosphatase (Boehringer Mannheim).

Methods

Fluorometric ST assay. For determination of secreted ST, 1 × 10^7 cells/mL were incubated in phosphate-buffered saline (PBS), pH 7.5, at 37°C for the times indicated. Culture supernatants were centrifuged at 800g to remove residual cells; the supernatant of a subsequent centrifugation at 2000g for 15 minutes was taken for determination of ST activity. The following glycoproteins were used as acceptors: 3 mg/mL asialofetuin, 1.6 mg/mL asialo-a2,3-acetyl glycoprotein, and 3 mg/mL asialomucin. The assay was performed using the respective hybridoma 1B2-1B7 obtained from the American Tissue Culture Collection (Rockville, MD). All MoAbs against carbohydrate epitopes used here are of IgM isotype.

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The linearity and of ST was as indicated above. The amount of lactose isomers sialylated in α2,6- or α2,3-linkage by the respective ST with 9-AMCA-surface fluorescence labeling was measured by flow cytometry as a fluorescent high-performance liquid chromatography (HPLC) system based on the system described previously. Isomers were separated on an amino propyl-phase column (25 × 0.4 cm) run isocratically with 15 mmol/L KH2PO4/acetonitrile (25/75, vol/vol) at 4 mL/min. The column efficiency was monitored using an Häch- F-1000 spectrophotometer (excitation 345 nm, emission 445 nm; Merck, Darmstadt, Germany). The fluorescent lactose isomers sialylated in α2,3- or α2,6-linkage were identified and quantified with respect to corresponding external standards that were prepared using the assay described above with commercially available α2,6-ST and α2,3-ST (Boehringer Mannheim) as the enzyme source. The retention time for the α2,3 isomer was 6.6 minutes and that of the α2,6 isomer was 8.7 minutes.

Flow cytometric detection of cell surface sialylation. For cytometric detection of surface sialylation by endogenous, extracellular ST and exogenous ST of rat liver, cells were preincubated in serum-free medium (PFHM II) for 48 hours. We found that fetal calf serum used for cell culture contains ST activity (approximately 2 µU/mL) that may accumulate at the cell surface. Although the influence of serum ST was minor and only clearly visible in cells with constitutively low ST activity such as IM-9 and Nalm-6, we preferred short-term culture in serum-free medium before the test to avoid background activity of serum ST. Culture of cells in serum-free medium neither changed viability nor proliferative activity compared with cells cultivated in medium with 5% fetal calf serum.

The cells were washed with PBS and then either treated with or without VCN (5 mU/1 × 10⁶ cells/100 µL PBS) for 30 minutes at 37°C. Cells (1 × 10⁷/100 µL PBS) were incubated for times indicated at 37°C with the addition of either CMP-5-F-Neu or CMP-9-F-NeuAc at 25 µmol/L, representing the approximate saturating concentration (3-fold Kₘ value). In some experiments, exogenous α2,6 ST of rat liver was added at concentrations of 0.1 µmol/L and 1 µmol/L. In previous experiments, it was found that, during an incubation time of 120 minutes, viability of cells was greater than 95% regardless of whether PBS or RPMI 1640 medium was used as the incubation medium. We always used PBS as the incubation medium.

For cytometric analysis using a FACScan cytometer (Becton Dickinson, Mountain View, CA), cells were washed twice and the cell pellet resuspended in RPMI 1640 medium containing 1 µg/mL propidium iodide. Green fluorescence of 1 × 10⁶ viable cells was analyzed by excluding dead cells identified by red fluorescence (propidium iodide label). For kinetic experiments, fluorescence intensity was determined as the mean fluorescence of fluorescent NeuAc per stained cell, calculated by the Lysis II software program (Becton Dickinson). As background values, we used the fluorescence of cells incubated at 0°C with fluorescent CMP-glyeosides or incubated at 37°C in the additional presence of CTP (2 mmol/L).

Possible effects of intracellular recycling of membrane glycans on surface sialylation were examined by blocking internalization at a reduced incubation temperature. First, JOK-1 cells were desialylated by treatment with VCN (50 mU/mL) for 2 hours at 8°C. VCN was removed by washing the cells and the cells were subsequently incubated for 3 hours at 8°C in the presence of CMP-5-F-Neu, as an example of corresponding controls additionally contained 2 mmol/L CTP. Aliquots were harvested after appropriate times and surface fluorescence labeling was measured by flow cytometry as described above.

Also, JOK-1 cells were fixed in various concentrations of glutaraldehyde (0.01%, 0.02%, and 0.05%) for 1 minutes on ice, quenched in 0.2 mol/L glycine for 5 minutes on ice, and washed three times in PBS before application in the cytometric sialylation assay as described above.

To further investigate involvement of membrane trafficking in surface sialylation, we treated desialylated JOK-1 cells with various doses of chloroquine (20, 50, and 100 µmol/L) for 45 minutes at 37°C. Sialylation of intact cells in the presence of CMP-5-F-Neu and chloroquine in the respective concentrations was performed at 37°C for 1 hour. Surface fluorescence labeling was measured by flow cytometry. Desialylated JOK-1 cells were also treated with brefeldin A at various concentrations (2, 5, and 10 µmol/L) for 45 minutes at 37°C. Cell surface sialylation as described above was performed in the presence of brefeldin A in the respective concentrations.

To determine whether cell surface-located ST is able to sialylate carbohydrate structures on other adjacent cells, human erythrocytes (Sangoncell S; Behring Werke, Marburg, Germany) desialylated by VCN were cocultivated with JOK-1 cells. Label transfer was assayed as follows. JOK-1 cells (1 × 10⁶ cells/mL) and desialylated erythrocytes (5 × 10⁵ cells/mL) were cultivated in the presence of 25 µmol/L CMP-5-F-Neu for up to 20 hours at 8°C. The fluorescence transferred to the cell surfaces was determined by flow cytometry using different instrument settings and electronic gates (JOK-1 cells: FSC/SSC linear mode; erythrocytes: FSC/SSC logarithmic mode).

Resialylation of defined surface antigens and glycan structures. To monitor the capacity of ecto-ST to resialylate MoAb-defined structures, we incubated VCN-treated JOK-1 cells together with 25 µmol/L CMP-5-F-Neu for 2 hours at 37°C. Cells were washed and lysed for 15 minutes at 0°C in lysis buffer that consisted of PBS containing 1% Triton X-100 and 1 mmol/L each of phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), and aprotinin. The cell lysate was centrifuged in an Eppendorf centrifuge for 15 minutes at 0°C. The supernatant was then taken for unspecific absorption of cellular proteins and specific immunoprecipitation as described previously. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blotting. Western-blotted proteins were incubated overnight at 4°C in PBS containing 3% bovine serum albumin (BSA). Incubation with sheep anti-FITC IgG (Fab-fragment) coupled to alkaline phosphatase diluted 1:1,000 in PBS containing 1% BSA was performed for 1 hour at room temperature. Immune reaction was visualized by using substrates NBT and BCIP.

Resialylation of glycan structures by ecto ST was examined by using MoAb against defined α2,6 sialylated glycan epitopes. For this, VCN-treated or untreated JOK-1 cells were incubated in the presence of 400 µmol/L CMP-NeuAc for 2 hours at 37°C. In some experiments, rat liver α2,6 ST was added (8 µU/200 µL) for the same incubation time. For each step of the experiment (ie, untreated cells, VCN-treated cells, and resialylated cells with and without addition of exogenous ST), indirect immunofluorescence of MoAb-labeled cells was measured by flow cytometry.

Confocal laser scanning microscopy. For detection of surface sialylation at the microscopic level, 5 × 10⁵ cells/reaction field were applied to Bio-Rad adhesion slides (Bio-Rad, Hercules, CA). Slides were pretreated according to the producer’s information. Cells were fixed in 3.7% (wt/vol) paraformaldehyde in PBS containing 2% (wt/vol) sucrose. Laser scanning microscopy was performed with a Zeiss confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) working with the blue line (488 nm wavelength) of an argon laser. Oil immersion plan neofluar lenses were used for confocal epifluorescence and for Nomarski differential interference contrast (DIC).

Indirect immunofluorescence of B-cell differentiation antigens.
Table 1. Fluorometric Determination and Characterization of ST Activity in Culture Supernatants of Human B-Cell Lines

<table>
<thead>
<tr>
<th>Activity (uU/mg)</th>
<th>Asialo-α,Acid gp</th>
<th>Asialomucin</th>
<th>α2,3 Isomer</th>
<th>α2,6 Isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nalm-6</td>
<td>0.4</td>
<td>&lt;0.1</td>
<td>19</td>
<td>91</td>
</tr>
<tr>
<td>JOK-1</td>
<td>4.0</td>
<td>&lt;0.1</td>
<td>4–9</td>
<td>91–96</td>
</tr>
<tr>
<td>U266</td>
<td>17.0</td>
<td>&lt;0.15</td>
<td>&lt;4</td>
<td>96</td>
</tr>
<tr>
<td>IM-9</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>

Product formation was linear with time (for at least 2 hours). The values obtained without acceptor glycoprotein or without lactose were below the detection limit and corresponded to the CTP reference.

* ST activity was determined by incorporation of 9-F-NeuAc into the acceptors asialo-α, acid glycoprotein for N-glycosylation and asialomucin for O-glycosylation, as described in the Materials and Methods.

† Lactose was used as acceptor and CMP-9-AMCA-NeuAc as donor substrate as described in the Materials and Methods. Isomers of sialylated lactose were separated by HPLC.

Indirect immunofluorescence staining of B-cell surface antigens was performed as previously described. As a second antibody, we used goat antimouse IgG + IgM(Fab) conjugated to HTC. Samples were measured in a FACScan cytometer (Becton Dickenson, Heidelberg, Germany).

RESULTS

B-Cell Lines Release ST Activity Into Culture Medium

The overall activity of ST found in culture supernatants was measured using CMP-[3H]-NeuAc as donor and asialofetuin as external acceptor according to Bauvois et al. ST activity released by the cells into the buffer during the 1 hour of incubation was sufficient to sialylate asialofetuin to various extents. Conditioned buffer of JOK-1 and U266 cells contained more ST activity than that of Nalm-6 and IM-9 cells (data not shown).

To determine the linkage specificity of the low ST activities found in culture supernatants, we performed a newly established fluorometric assay using lactose as acceptor and CMP-9-AMCA-NeuAc as donor (Table 1). Isomers of sialylated lactose were subsequently separated by HPLC. All cell lines with the exception of IM-9 showed a propensity for α2,6 ST. The percentage of α2,6 isomer of the lactose substrate was significantly higher than that of the α2,3 isomer when ST activity of culture supernatants was measured (Table 1). IM-9 cells were totally deficient of α2,6 ST activity.

The fluorometric method for determination of ST activity developed previously allows us to differentiate the acceptor specificity of ST even at low enzyme activities. We examined whether the ST activity secreted by the cell lines preferentially sialylates N- or O-linked glycoconjugates (Table 1). Fluoresceinated NeuAc was incorporated into asialo-α, acid glycoprotein, a protein with defined N-glycosylation, in larger quantities in comparison to the exclusively O-glycosylated asialomucin. U266 cells showed highest ST activity in culture supernatants. IM-9 cells exclusively synthesized ST with α2,3 linkage specificity and exhibited incorporation of labeled NeuAc into asialomucin. Overall, ST activity in the culture supernatant was very low in Nalm-6 and IM-9 cells. Because viability of cells during the assays was greater than 95%, ST activity found in the supernatants did not result from cellular leakage.

Cell Surface Glycoconjugates Are Sialylated by Extracellular ST Activity

We next asked whether extracellular activity is able to sialylate cell surface glycoconjugates. This approach afforded a new sensitive method to detect very low activities on cell surfaces. To this end, the cells of the four cell lines were washed three times in PBS and then incubated in the presence of CMP-5-F-Neu. The incubation was stopped at various times and the fluorescent NeuAc incorporated into cell surface molecules of live cells was quantitatively assessed using flow cytometry.

The kinetic experiments showed that all four cell lines were able to sialylate cell surface structures by an endogenous, extracellular ST activity when fluorescent CMP-sialic acid was offered as donor (Fig 1). The standard assay was...
performed with CMP-5-F-Neu due to its superior kinetic properties and its high resistance to sialidase. Without prior VCN treatment of the cells, the incorporation rate of 5-F-Neu was slow in JOK-1 and IM-9 cells and remained at basal levels in Nalm-6 and U266 cells. However, when cells were treated with VCN before the labeling procedure, a significant increase of the 5-F-Neu incorporation rate was observed. A high increase was found in JOK-1 and U266 cells, in contrast to a markedly slower rate in Nalm-6 and IM-9 cells. The kinetics of surface sialylation of JOK-1 and U266 cells after VCN-treatment were almost linear during the 2 hours of incubation. The increased incorporation of 5-F-Neu into surface molecules of JOK-1 and U266 cells after VCN treatment indicates that untreated cells express large amounts of sialylated glycans. The differences in fluorescence intensity with and without VCN treatment were smallest in IM-9 cells. This points to the fact that IM-9 cells express fewer sialylated surface structures than the other cell lines.

Surface labeling with fluorescent NeuAc was a temperature-dependent reaction, because no significant label was incorporated when the cells were kept at 0°C for 2 hours. The enzymatic origin of surface sialylation was confirmed by two approaches. (1) Incorporation of fluoresceinated NeuAc was completely inhibited when either unlabeled CMP-NeuAc was added in a 20-fold excess (final concentration, 0.5 mmol/L) or CTP in a 80-fold excess (final concentration, 2 mmol/L). (2) Neuraminidase (VCN) treatment for 30 minutes of cells labeled with 9-F-NeuAc reduced the label by approximately 60% of viable cells that were treated either with or without VCN before the labeling procedure (Fig 2). For this approach, CMP-9-F-NeuAc was applied in the surface sialylation assay because it can be released by sialidases after enzymatic transfer to the oligosaccharide chain. However, cleavage of 9-F-NeuAc proceeds at a reduced rate as compared with parent NeuAc. The efficiency of the VCN-mediated cleavage was monitored by complete abrogation of CDw75 and HB-6 expression (see Fig 8). This result also shows that fluorescently labeled acceptor structures are to a large extent accessible for exogenous sialidase. Sialidase-resistant CMP-5-F-Neu was used for most surface sialylation experiments instead of CMP-9-F-NeuAc to avoid the effects of sialidases possibly present in the assay system.

The protease sensitivity of fluorescently labeled cell surface acceptor structures was assayed as follows. JOK-1 cells were incubated in the presence of CMP-5-F-Neu (25 μmol/L) for 2 hours at 37°C, subsequently washed, and further incubated with 0.25% trypsin in PBS for 10 minutes at 37°C. Surface-bound fluorescence of live cells was reduced by 50%, as determined by flow cytometry. Further support for surface location of acceptor structures was obtained by the quenching effect of an antifluorescein antibody; 5-F-Neu labeled JOK-1 cells were incubated together for 5 minutes at 4°C with an mouse antifluorescein MoAb (dilution 1:20) described to efficiently quench FITC-fluorescence emission. The antibody quenched surface fluorescence by 60% as determined by flow cytometry.

Cell surface sialylation was also shown by confocal laser scanning microscopy of 5-F-Neu-labeled cells (Fig 3). As also observed by flow cytometry, fluorescence intensity was weaker without VCN pretreatment of cells (data not shown). Fluorescence appeared in dot-like, clustered formations at the cell surface (Fig 3A). Interestingly, not all cells were stained with equal intensity; some were almost negative or staining intensity was less than the detection limit. In accordance with this result, flow cytometric analysis also showed a wide distribution of 5-F-Neu label intensity in the cell populations ranging from almost negative to strongly positive cells (limit values of mean intensity between 50 and 1,000).

Cell Surface Sialylation Is Mediated by Ecto-ST

We investigated whether soluble ST found in culture supernatants mediates surface sialylation. Surface sialylation followed a linear course, as outlined above (Fig 1). In contrast, when conditioned culture supernatant of U266 and JOK-1 cells was taken at several time points and ST activity was determined by applying an external acceptor protein (asialo-α-L-acid glycoprotein), fluorescence intensity was increasing. Apparently U266 and JOK-1 cells released increasing amounts of ST during an incubation time of 90 minutes (Fig 4).

![Fig 2. Flow cytometric analysis of 9-F-NeuAc-labeled JOK-1 cells with and without cleavage of NeuAc by neuraminidase treatment (VCN). Row A, without prior VCN treatment; row B, with prior VCN-treatment (30 minutes). Line 1, control treatment at 0°C; line 2, 9-F-NeuAc-labeled cells after 60 minutes of incubation with CMP-5-F-NeuAc; line 3, 9-F-NeuAc-labeled cells subsequently treated with VCN for 30 minutes.](www.bloodjournal.org)
Fig 3. Confocal laser scanning microscopy of JOK-1 cells incubated with CMP-5-F-Neu for 60 minutes and sialylation by ecto-ST. Cells were treated with VCN (30 minutes) before labeling. (A) Surface immunofluorescence of JOK-1 cells. Weak fluorescence shadowing into the cytoplasm is unspecific and caused by pixel noise. (B) Differential interference contrast image of cells shown in (A).

This observation gave rise to the question of whether intensity of surface sialylation is dependent on the amount of ST activity secreted. To test this, we added conditioned media of various preincubation times to washed, fresh cells and incubated them in the presence of CMP-5-F-Neu for an additional 30 minutes. Conditioned culture supernatant, even after 90 minutes of preincubation, did not enhance surface sialylation compared with unconditioned medium during a reaction time of 30 minutes. The same results were obtained regardless whether JOK-1, U266, or IM-9 cells were applied in this assay. As an example, data for cell line U266, which secreted the highest amounts of ST, are depicted in Table 2. From these results we conclude that the concentration of ST in the culture medium does not influence the rate of surface sialylation. Obviously, secreted ST activity does not contribute to the sialylation of cell surface acceptors. In further support of this assumption, the addition of exogenous acceptor (asialo-α1-acid glycoprotein or asialofetuin) to either desialylated or untreated JOK-1 cells incubated together with CMP-5-F-Neu did not significantly reduce surface sialylation (Table 3).

Surface sialylation is apparently mediated by a surface-expressed ecto-ST different from that found in the culture supernatant. The efficiency of endogenous, ecto-ST activity was shown when high amounts of exogenous ST activity

<table>
<thead>
<tr>
<th>Precondition Time (min)</th>
<th>VCN Pretreatment</th>
<th>Mean Fluorescence Intensity</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>--</td>
<td>39</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>--</td>
<td>36</td>
</tr>
<tr>
<td>90</td>
<td>+</td>
<td>278</td>
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Conditioned culture supernatant of U266 cells from various incubation times was added to washed, fresh U266 cells with or without VCN pretreatment. Surface sialylation was performed in the presence of 25 μmol/L CMP-5-F-Neu for 30 minutes and measured by flow cytometry.
were applied to the cells. Exogenous α2,6 ST of rat liver at a concentration of 10 μU/mL induced a twofold increase in surface sialylation (Table 4), but only after pretreatment of cells with neuraminidase. Apparently, exogenously added ST is not able to significantly improve the sialylation extent achieved by the endogenous ecto-ST.

The question of whether secreted and ecto-ST have the same characteristics concerning acceptor specificity cannot be answered at the moment. The same donor Km value of approximately 8 μmol/L for CMP-5-F-Neu was determined for the secreted and surface-located enzyme of U266 cells (Fig 5).

To investigate whether surface-located ST is a ligand for other surface molecules or exists as a membrane-integrated protein, we incubated VCN-treated and untreated JOK-1, U266, and IM-9 cells in acidified RPMI 1640 medium (pH 3) for 5 minutes before the flow cytometric enzyme assay. Cells were washed two times with PBS and incubated with CMP-5-F-Neu for 30 minutes at 37°C. Incubation in acidified medium that has been shown to release receptor-bound ligands from the cell surface did not affect incorporation of fluorescent NeuAc. If ST were bound to a surface receptor, one would expect at least a diminished fluorescent intensity by this treatment compared with untreated cells. This result indicates that ecto-ST activity of B lymphocytes is not bound to surface components as a secreted product but rather exists as membrane-integrated enzyme. For the control, we also used IM-9 cells from cultures incubated in the presence of 5% fetal calf serum. In these cells, preincubation in acidified medium resulted in decreased incorporation of 5-F-Neu by about 60% (data not shown). This result further underlines that serum-derived ST may stick to the cell surface during cultivation in fetal calf serum-containing medium.

Surface Sialylation Is Not Mediated by Intracellular ST Activity

Hypothetically, desialylated surface proteins may be internalized and sialylation may occur by intracellular ST, followed by the reappearance of labeled sialoglycans at the cell surface. Because we observed a linear, continuous increase in fluorescent label after the addition of CMP-5-F-Neu at 37°C (Fig 1) without an apparent lag-phase, such an internalization process appears to be unlikely. We further investigated surface sialylation after blocking of internalization, and intracellular processing to answers this question unequivocally.

When surface sialylation of VCN-desialylated JOK-1 cells in the presence of CMP-5-F-Neu was performed at 8°C for 3 hours, a continuous increase of surface label was measured in flow cytometry (Fig 6), albeit to a much lower level than at 37°C (after 2 hours of incubation: mean fluorescence 180 (8°C) compared with mean fluorescence 2070 (37°C); for comparative cytometric measurement, the fluorescence amplification was reduced by 7-fold in relation to data shown in Fig 6). At the reduced incubation temperature, membrane
traffic is blocked completely. As already observed, at 37°C, sialylation reaction also started without lag phase.

Treatment of cells with glutaraldehyde at concentrations of 0.05% for 30 seconds has been shown to block internalization. When JOK-1 cells fixed with glutaraldehyde at incremental concentrations (from 0.01% to 0.05%), fluorescent label was incorporated during an assay time of 60 minutes at 37°C using CMP-5-F-Neu as substrate. The label obtained was reduced in its intensity as compared with untreated cells (at 0.02% glutaraldehyde, the fluorescence mean intensity was reduced by 53%; at 0.05% glutaraldehyde, the fluorescence mean intensity was reduced by 52%). The fixation process probably affects activity of surface ST. These results strongly indicate that, under the given experimental conditions, fluorescent label is transferred by a surface-located ST that is affected in its activity by the fixation.

Because cell surface sialoglycans may passage the lysosomal compartment en route to the Golgi compartment during their recycling pathway, we applied the lysosomotropic amine chloroquine to impair functions of this acidic organelle. VCN-desialylated JOK-1 cells treated with chloroquine at various concentrations for 45 minutes were assayed for their capacity to perform surface sialylation. After 60 minutes of incubation in the presence of chloroquine and CMP-5-F-Neu, chloroquine-pretreated cells did not show decreased incorporation of fluorescent label into surface glycans in comparison to untreated cells (Table 5).

The fungal lactone brefeldin A is an inhibitor of membrane transport. In particular, it causes disassembly of Golgi structures and also affects the organization of lysosomes, endosomes, and trans-Golgi network (TGN). We incubated JOK-1 cells together with brefeldin A at various concentrations for 45 minutes, followed by VCN treatment before the sialylation assay (60 minutes). No effects of brefeldin A on surface sialylation were observed (Table 5).

In summary, the blockade of internalization, disruption of TGN structures, and impairment of intracellular processing pathways did not inhibit surface sialylation. From these data, we suggest that surface sialylation of glycans on human B cells under the given experimental conditions is a process mediated by a surface-located ST that does not require intracellular recycling of any of the components involved.

**Table 5. Effects of Chloroquine and Brefeldin A on Surface Sialylation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µmol/L)</th>
<th>VCN Pretreatment</th>
<th>Mean Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>84</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>20</td>
<td>+</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>+</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>101</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>+</td>
<td>58</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>2</td>
<td>+</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>461</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td>457</td>
</tr>
</tbody>
</table>

JOK-1 cells with or without VCN pretreatment were incubated in the presence of chloroquine/brefeldin A at the concentrations indicated for 45 minutes at 37°C. Surface-bound fluorescence was measured by flow cytometry. Data for chloroquine and brefeldin A treatment were generated in two independent experiments.
The four cell lines investigated express various α2,6 sialylated carbohydrate structures that are recognized by MoAbs.10 The expression of some of these epitopes is listed in Table 6. Recognition of these epitopes can be abolished by treatment of the cells with VCN before antibody staining.

Interestingly, MoAb 1B2, which recognizes nonsialylated lacto-N-glycosyl type II carbohydrate chains (Galβ1-4GlcNAcβ1-R), did not react with surface-exposed epitopes on the four cell lines studied (Table 6). Only after desialylation did 1B2 epitopes become visible on the cell surface. Consequently, cell line IM-9, which is deficient for sialylated carbohydrate sequences. For this purpose, JOK-1 cells were desialylated by VCN treatment and incubated in the presence of unlabeled CMP-NeuAc with and without the addition of external α2,6 ST derived from rat liver. Cells were stained with MoAb against CDw75, CDw76, HB-4, HB-6, and the 1B2 epitope. Antibody reactions were measured by flow cytometry. As can be seen in Fig 8, VCN treatment abolished reactivity with those antibodies dependent on α2,6 sialylated carbohydrate epitopes (CDw75, CDw76, and HB6) and increased reaction with MoAb 1B2, which detects nonsialylated N-acetyl-lactosamine groups. Resialylation with ecto-ST activity let the CDw75 and the HB6 epitopes reappear at the cell surface and, consequently, the 1B2 epitope diminish in its expression. The CDw76 epitope remained unsialylated by this treatment. Exogenous rat ST only slightly enhanced reappear-ance of the CDw75 epitope and, more significantly, that of the HB-6 antigen compared with endogenous ecto-ST. Under the experimental conditions described, CDw75 could be resialylated by 11% (endogenous ST) and 17% (exogenous ST) and HB-6 by 18% (endogenous ST) and 48% (exogenous ST). When alterations in 1B2 expression, representing N-acetyllactosamine groups, were taken to assess resialylation efficiency, approximately 33% for endogenous and 70% for exogenous ST activity was calculated.

In kinetic experiments, a continuous increase in resialylation of the surface-expressed HB-6 epitope was detected (Fig 9). Although the time course implied a lag phase of approximately 10 minutes that is most likely caused by statistical deviation of low mean fluorescence values, this experiment is in accordance with the linear increase of fluorescent label as shown in Figs 1 and 6. Incubation without the addition of exogenous CMP-NeuAc did not result in resialylation of HB-6, thus excluding effective sialylation of HB-6 by regular glycoprotein turnover. The same result was be verified by using an extended panel of MoAbs directed against α2,6 sialylated sugar sequences.

Table 6. Sialylated Cell Surface Antigens of Human B-Cell Lines

<table>
<thead>
<tr>
<th>Antigen (with or without VCN pretreatment)</th>
<th>Nalm-6</th>
<th>JOK-1</th>
<th>U266</th>
<th>IM-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24*</td>
<td>++</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>CDw75</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CDw76 (+)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>HB-4 +St</td>
<td>-</td>
<td>+S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HB-6 ++</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IB2†</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

* From the panel of CD24 MoAb, only MoAb VIB-E3 was found to be reactive with a sialoglycan epitope.
† Subpopulation of cells reactive.
‡ MoAb 1B2 detects the nonsialylated carbohydrate sequence Galβ1-4GlcNAcβ1-R.
Relative Fluorescence

Fig 8. Resialylation of antibody-defined oligosaccharide structures by exogenous and endogenous ecto-ST activity. At each step of the experiment, JOK-1 cells were incubated with MoAb directed against carbohydrate epitopes: CDw75 (antibody HH2), CDw76 (antibody HD66), HB-6, and 1B2. Staining was performed with antimouse IgG + IgM antibody conjugated to FITC and cell surface-located fluorescence was determined using flow cytometry. The mean fluorescence is indicated for each histogram. Cells were stained before VCN treatment, after VCN treatment (+VCN), after incubation with endogenous ST (+ectoST), and with the addition of external α2,6 ST of rat liver (+ext.ST). VCN treatment was performed under standard conditions, as described, for 30 minutes with $6 \times 10^6$ cells/600 µL PBS. Cells were extensively washed and further incubated in 600 µL RPMI 1640 medium with the addition of CMP-NeuAc at a final concentration of 400 µmol/L and 150 µmol/L 2,3-dehydro NeuAc for 120 minutes. To determine the effect of exogenous α2,6 ST, α2,6 ST of rat liver was added at a concentration of 10 mU/mL together with 30 µg of BSA. Control incubations were performed without the addition of CMP-NeuAc (data not shown).

obtained with CTP in excess (Fig 9). Successful resialylation of the CDw75 and HB-6 epitopes indicates that the ecto-ST activity of B cells is specific for α2,6 linkage.

Ecto-ST of B Cells Does Not Sialylate Surface Glycans of Neighboring Erythrocytes

Because surface-located ST has been discussed to mediate contacts to other cells by sialylating their surface structures,¹ we investigated whether ecto-ST of B cells is able to sialylate neighboring cells. For this purpose, we incubated JOK-1 cells together with desialylated erythrocytes in the presence of CMP-5-F-Neu for up to 20 hours at 8°C and measured the fluorescent surface label of erythrocytes. Erythrocytes have been reported to express CDw75 and HB-6, those structures that can be resialylated by ecto-ST (Fig 8).¹³ No significant label was measured when desialylated erythrocytes were incubated alone, because erythrocytes neither express ecto-ST nor secrete ST activity.³ Also, when desialylated erythrocytes were incubated together with JOK-1 cells, no fluorescent label was observed on erythrocytes gated by their specific scatter signals in flow cytometry. Surface sialylation of cocultivated JOK-1 cells was not reduced. However, rat liver α2,6 ST is able to transfer fluorescent sialic acid to erythrocyte surface glycans.³² The failure to transfer label to erythrocytes further speaks against interference by a soluble ST.
SIALYLTRANSFERASE OF HUMAN B CELLS

In a recent study, normal activated B cells were found to express more α2,6 ST transcripts than mature stages of B cells, which points to increased production of α2,6 ST during B-cell activation. Although extracellular ST activity has been reported for other cell lines, e.g., murine melanoma and B-cell lines33,34 and rat hepatoma cells,35 and increased levels of ST have been found in plasma of cancer patients,36 a physiologic function of released ST activity is still unclear. Sialylation of cell surface structures by addition of exogenous CMP-NeuAc has been described.30-35 These activities may either originate from secreted or cell surface ecto-ST. An unequivocal differentiation between these two forms of ST was not yet provided. To date, there are only few studies pointing to the existence of a membrane-located α2,6 ST. In an earlier report, it was shown by radiometric assays and electron microscopic autoradiography that intracellular ST activity of murine leukemic L1210 cells is able to sialylate cell surface structures. Plasma membrane-located ST was observed on intestinal cells by immunoelectrochemical techniques.28 On B lymphocytes, we and others could not detect surface-expressed ST by means of antibodies directed against α2,6ST.12,13 We reasoned that immunostaining may be too insensitive to detect ecto-ST on lymphocytes. As shown here, surface ST of B lymphocytes could only be detected by its enzymatic activity. The enzymatic assay system we used amplifies the fluorescent detection to a great extent, thus resulting in a higher sensitivity compared with fluorescent antibody reactions.

We observed that all cell lines tested incorporated fluorogenic sialic acid into cell surface molecules without the addition of exogenous ST activity. The reaction was significantly increased when sialidase-treated cells were used for the assay. Surface sialylation was specific because the incorporation of labeled NeuAc could be inhibited by the addition of unlabeled CMP-NeuAc or CTP and by incubation at 0°C. Moreover, fluorescein-labeled, sialylated glycoproteins could be visualized in Western blots. The reaction affected surface-expressed molecules because incorporated fluorescent label could be drastically reduced by subsequent VCN treatment of live cells, as shown by flow cytometric analysis. Fifty percent of fluorescently labeled acceptors were sensitive to mild trypsinization. The resistant fraction may be, at least in part, composed of glycosphingolipids.9

Surface sialylation was not influenced by the soluble ST activity because conditioned media did not significantly enhance sialylation compared with the 30 minutes of incubation with unconditioned medium. This finding strongly pointed to a sialylation mediated by an ecto-ST different to soluble ST. In addition, the kinetics of surface sialylation differed from those of supernatant ST when the external acceptor was applied. Also, ecto-ST seems to be a membrane-integrated enzyme because treatment to release noncovalently attached molecules from the cell surface remained ineffective.

Several observations speak against a sialylation of recycled glycans by intracellular ST. (1) In kinetics of fluorescent label incorporation, no lag-phase was measured. For internalization of surface glycoproteins and subsequent resialylation by intracellular ST, one would expect a distinct lag-phase. (2) When internalization was blocked either by lowered incubation temperature (8°C) or by glutaraldehyde fixation, ST activity was determined, albeit at reduced rates. Because
ecto-sialylation is a temperature-dependent process, reduced activity at 8°C was expected. Glutaraldehyde fixation greatly reduces the mobility of surface proteins or affects protein structure, thus influencing the enzymatic activity. (3) Chloroquine and breveldin A, substances that alter intracellular trafficking, showed no effect on the intensity of surface sialylation when CMP-F-NeuAc was added to these cell cultures.

Also, because CMP-F-NeuAc is a membrane impermeable probe, sialylation of surface glycoportins with fluorescent substrate must take place outside. In an earlier study, intracellular trafficking of cell surface sialglycoconjugates has been investigated. It was found that the bulk of sialoglycans do not recycle to intracellular organelles with ST activity. Thus, there is ample evidence that surface sialylation as measured here is a result of a surface-located ST. Comparing ecto-ST with exogenously added rat liver ST, an increase in sialylation by exogenous ST was only observed on VCN-treated cells when the exogenous enzyme was applied in high excess. This could mean that either galactose acceptor sites are better accessible to ecto-ST than to exogenous ST from rat liver or that acceptor fine specificity of both enzymes is different.

Cell surface ST was able to resialylate major cell surface glycoproteins of B cells such as HLA class I and II, the transferrin receptor, and surface IgM. Moreover, surface α2,6 sialylglycan structures CDw75 and HB-6 were resialylated in the presence of CMP-NeuAc. Other antigens of this group, such as CDw76 and HB-4, remained unreactive with the respective MoAbs after desialylation. Steric hindrance or structural modifications, such as fucosylation or different branching of the respective oligosaccharides, may impede accessibility of acceptor sites in these molecules. In contrast to resialylation by ecto-ST, terminal glycosylation in the Golgi compartment is mediated by concerted and competitive action of several glycosyltransferases. On the other hand, selective sialylation may be a result of enzyme fine specificity. The superior resialylation efficiency of distinct surface structures (HB-6 and CDw75) achieved by surface active ST compared with exogenous ST from rat liver is in accordance with the observation published previously that surface ST, although adding back only a small percentage of total surface sialic acid, specifically restored structures involved in mixed lymphocyte reactions. Thus, although only present in small numbers, surface ST is able to resialylate distinct surface structures to a great extent. Moreover, the use of carbohydrate-specific antibodies and antibodies against protein structures shows that, although only distinct carbohydrates are resialylated by ecto-ST, most functionally important surface antigens are reached by the enzyme. Because the epitopes recognized by CDw75 and HB-6 require sialic acid in α2,6 position, these experiment provide further proof that ecto-ST of human B cells is an α2,6 ST.

Ltk− cells transfected with the human α2,6 ST gene convert from negative to high expression of CDw75 at the cell surface. On these transfecants, only very low levels of surface ST activity could be detected by flow cytometry using fluorescent CMP-NeuAc as the donor substrate. Furthermore, CDw75 could not be reconstituted on VCN-pre-treated Ltk− transfectants in the presence of exogenous CMP-NeuAc (unpublished results). These experiments confirm that overexpression of Golgi-located α2,6 ST is not necessarily associated with surface expression of the corresponding ST activity.

A major reservation against a physiologic role of ecto-ST was the question of whether CMP-NeuAc as substrate is available in the extracellular milieu. However, several studies describe the presence of sugar nucleotides in extracellular fluids. CMP-NeuAc was found in human serum and extracellular UDP-Gal accessible to cell surface galactosyltransferase was given. Although concentrations of serum CMP-NeuAc were found to be low, it should be considered that ecto-ST of B cells was efficiently acting at low donor substrate concentrations (Fig 5).

Ecto-ST may have a physiologic role as an adhesion molecule in cell-cell interactions, as has been shown for the galactosyltransferase. To examine this possibility, we incubated desialylated erythrocytes together with JOK-1 cells and CMP-F-NeuAc. Although erythrocytes express CDw75 and HB-6, no fluorescent label was transferred to erythrocytes under these experimental conditions. The surface ST apparently requires very close contact for enzymatic activity that may be only provided by surface glycans on the same cell. We tentatively conclude that ecto-ST of B cells is not primarily involved in cell to cell interaction. It was recently shown that the CD22 lectin interacts with the CDw75 epitope during B-B-cell adherence. Ecto-ST may have a task in α2,6 sialylation of ligand structures on the cell surface, thereby regulating adhesion processes.

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REFERENCES

lymphocyte differentiation antigen CD76 bind to gangliosides. FEBS Lett 261:347, 1990
34. Miller DJ, Macak MB, Shur BD: Complementarity between sperm surface β1,4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. Nature 357:589, 1992
35. Begovic PC, Shi YY, Mansfield D, Shur B: Evidence that cell surface β1,4-galactosyltransferase spontaneously galactosylates an underlying laminin substrate during fibroblast migration. J Biol Chem 269:31793, 1994
43. van Boxel JA: IgD on cell membranes of human lymphoid cell lines with multiple immunoglobulin classes. Nature 251:443, 1974
47. von Endert PM, Moldenhauer G: Inhibitory and stimulatory
Ecto-sialyltransferase of human B lymphocytes reconstitutes differentiation markers in the presence of exogenous CMP-N-acetyl neuraminic acid

HJ Gross, A Merling, G Moldenhauer and R Schwartz-Albiez