Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 30-40

ENZYMATIC ANALYSIS OF CELL SURFACE LACTOSAMINYL GLYCANS BY FLOW CYTOMETRY

L.C. Bollheimer, R. Schwartz-Albiez[#], A. Sauer and H.J. Groß[*]

Institut für Biochemie II, Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, FRG

[#] Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, FRG

Received August 22, 1995

Cell surface expressed lactosaminyl glycans were determined on live cells by flow cytometry using a sialyltransferase mediated labeling procedure. Fluorescent CMP-sialic acid and Galβ1,4GlcNAc α2,6-sialyltransferase were applied to probe expression of acceptor glycans on untreated or sialidase pretreated erythrocytes. After enzymatic fluorescence labeling, erythrocytes were treated with endo-β-galactosidase or trypsin to distinguish polylactosaminyl- and complex-type glycans. The expression of lactosaminyl sequences on cord- was 20% lower than on adult cells. After sialidase treatment fluorescence incorporation on both cell types increased twofold compared to untreated cells indicating a low sialylation extent. A recombinant α2,3-sialyltransferase was preferentially labeling polylactosaminyl glycans. Taking advantage of the different fine specificity as determined here, α2,6- and α2,3-sialyltransferase can be applied to distinguish certain types of lactosaminyl glycans.

Sialylated glycans derived from lactosaminyl precursor sequences were identified as differentiation antigens and ligands for adhesion proteins *(1-9)*. Terminal galactose is recognized by specific lectins found on the surface of macrophages and liver cells *(10,11)*. Several antigens on erythrocytes consist of lactosaminyl- and polylactosaminyl glycans, for example blood group antigens (ABO, Lewis), cold agglutination antigens, and the senescent antigen involved in erythrocyte sequestration *(12-14)*.

Definition of the glycosylation state of a particular subset of hematopoietic cells helps to understand the function of surface expressed glycans during maturation and activation. In order to analyse surface glycosylation of live cells we developed a cytometric assay based on the principle of exogenous enzymatic sialyltransfer *(15-18)*.

* **corresponding author:** Hans Jürgen Groß; fax: 06221-565586 (FRG).

Abbreviations:
CMP-NeuAc: Cytidine-5′-monophospho-5-N-acetyl-neuraminic acid; 9-fluoresceinyl-NeuAc: 5-N-acetyl-9-deoxy-N-(N′-fluoresceinyl)-thioureido-neuraminic acid; 5-fluoresceinyl-Neu: 5-(N-fluoresceinyl)-thioureido-acetyl-neuraminic acid.

0006-291X/95 $12.00

For this approach intact cells, native or desialylated by sialidase, were incubated together with CMP-activated fluorescent sialic acid and sequence specific sialyltransferase (ST). Labeled glycans were further characterized by endoglycosidases. Incorporated fluorescence was quantitatively determined by flow cytometry (for experimental procedure see diagram). This assay system was applied here to investigate maturation dependent expression of lactosaminyl sequences on erythrocytes. The method is useful to evaluate alterations of these glycans and of their sialylation state on live cells during differentiation under physiologic or pathologic conditions.

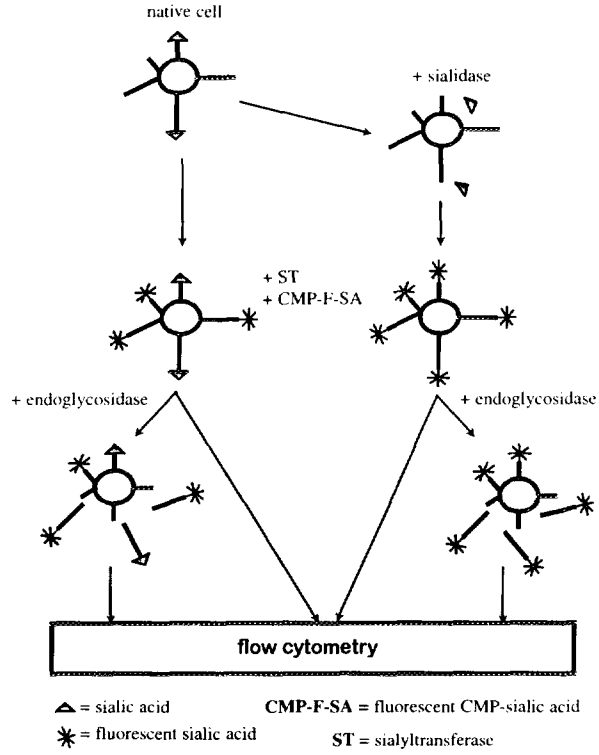
MATERIALS AND METHODS

Materials

BSA, Nonidet-P40, *rat liver* Galβ1,4GlcNAc α2,6-sialyltransferase (α2,6-ST; E.C. 2.4.99.1.), *trypsin* (E.C. 3.4.21.4), β-galactosidase from *Streptococcus pneumoniae* (E.C. 3.2.1.23), *Arthrobacter ureafaciens* (AU-)sialidase (E.C. 3.2.1.18) and 2,3-dehydro-NeuAc were supplied by Boehringer (Mannheim), endo-β-galactosidase from *Bacteroides fragilis* (E.C. 3.2.1.103) from Oxford GlycoSystems (Abingdon). Recombinant Galβ1,3(4)GlcNAc α2,3-sialyltransferase (α2,3-ST; E.C. 2.4.99.5) was supplied by CYTEL (San Diego; *19)*. CMP-5-fluoresceinyl-Neu and CMP-9-fluoresceinyl-NeuAc were prepared as described *(20-22)*;

Enzymatic sialylation of erythrocytes by fluorescent sialic acids

Adult human erythrocytes were collected from healthy donors by venipuncture (1 mg EDTA /ml blood). Alternatively, pooled erythrocytes (Sangocell, Behring Werke, Marburg, FRG) or



pooled cord erythrocytes (Biotestcell C, Biotest, Dreieich, FRG) were employed. Cells were suspended in cell incubation buffer (CIB; PBS pH 6.8 / 5 mg/ml BSA).

For desialylation, erythrocytes (5×10^8/ml) were incubated for 1h in CIB containing 100 mU/ml AU-sialidase; for selective cleavage of β1,4-linked galactose (23), the cells were incubated for 2h at 37°C in CIB with 50 mU/ml β-galactosidase from *S. pneumoniae*. Untreated or pre-treated erythrocytes were sialylated for different periods in CIB at 37°C. Standard assay contained 5×10^8 cells/ml, 50 µM CMP-5-fluoresceinyl-Neu, 100 µM 2,3-dehydro-NeuAc, and 60 mU/ml α2,3-ST or 40-60 mU/ml α2,6-ST, respectively. ST activity was measured as described *(22)*. To assess viability of erythrocytes, haemoglobin release was monitored as described *(24)*. After lysis of erythrocytes labeled with fluorescent sialic acid (0.5% Nonidet-P40), the fluorescence bound to macromolecules was determined as described *(22)*.

Erythrocytes labeled with fluorescent sialic acid (5.0×10^8/ml) were suspended in CIB pH 5,9 and incubated with 40 mU/ml endo-β-galactosidase at 37°C for 1h, or suspended in PBS and incubated with 200 µg trypsin/ml for 45 min at 37°C.

Flow cytometry of intact erythrocytes

Flow cytometric analysis of labeled erythrocytes was performed using a FACScan cytometer (Becton & Dickinson, Heidelberg, FRG). Routinely, 20,000 cells were aquired; photomultiplier for fluorescence, forward scatter (FSC) and sideward scatter (SSC) were adjusted to the log-mode. Data were analyzed by the Lysis II program (Becton & Dickinson) and fluorescence intensity was calculated as mean value per single cell.

RESULTS

Enzymatic labeling of erythrocyte surface glycans with fluorescent sialic acid

Figure 1a illustrates the time courses for the incorporation of either 5-fluoresceinyl-Neu or 9-fluoresceinyl-NeuAc to lactosaminyl glycans applying Galß1,4GlcNAc α2,6-ST. The mean fluorescence value per single cell was determined by flow cytometry from measurement of 20,000 erythrocytes. Cell debris, platelets and clustered cells were excluded by electronical gating of singular erythrocytes according to their forward- and sideward scatter signal. (Figure 1b). Within the population of erythrocytes incorporated fluorescence showed a logarithmic gaussian distribution with a variation coefficient of about 30±5% (FL-1 histogram in Fig. 1b). After 9 hs incubation at 37°C, surface labeling reached saturation. Final label achieved with CMP-5-fluoresceinyl-Neu on native cells was about 1,3fold higher than that with CMP-9-fluoresceinyl-NeuAc. Corresponding incubations in the absence of α2,6-ST gave no significant fluorescence. Addition of further enzyme after 9 h did not improve the final fluorescence value. Saturation was also achieved after an incubation at 8°C for 24 h. Labeling at low temperature is useful if membrane turnover has to be blocked (17).

Transferred fluorescence per single cell measured by flow cytometry (mean value) was correlated to the amount of fluorescent sialic acid incorporated (pmol per 10^6 cells or per mg protein) by using the chromatographic system described earlier *(21, 22)*. Following detergent lysis of the cells, fluorescence bound to macromolecules was quantified after gel filtration on Sephadex G-50 (Table 1). In order to prove surface restriction of the label and to exclude unspecific adsorption of fluorescence, erythrocytes

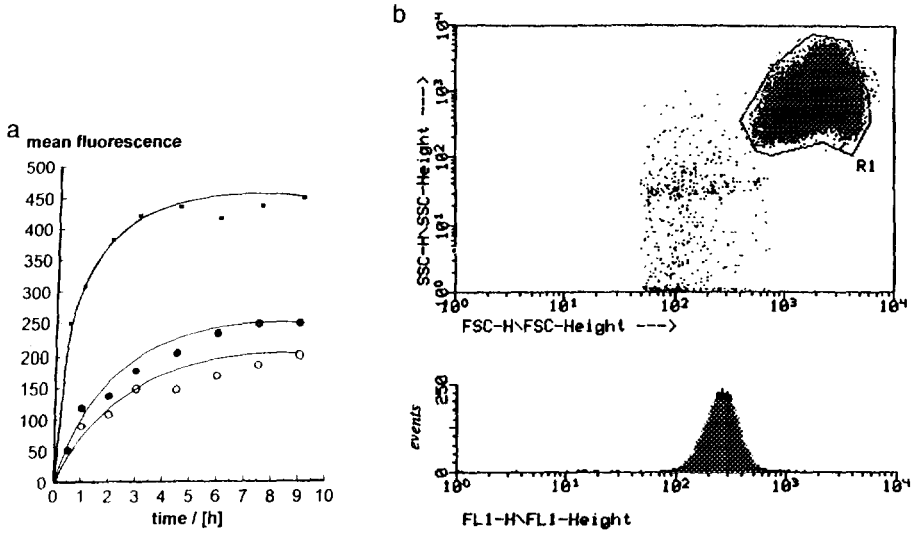


Figure 1.

a: Time courses for incorporation of 5-fluoresceinyl-Neu and 9-fluoresceinyl-NeuAc by rat liver Galβ1,4GlcNAc α2,6-sialyltransferase (40mU/ml) to adult erythrocytes as determined by flow cytometry.

- ■ - desialylated (AU-sialidase) adult erythrocytes; 50μM CMP-5-fluoresceinyl-Neu
- ● - adult native erythrocytes; 50μM CMP-5-fluoresceinyl-Neu
- O - adult native erythrocytes; 50μM CMP-9-fluoresceinyl-NeuAc

b: Electronical gating and fluorescence distribution examplified for a representative sample (9 h) from the time course depicted in Figure 1a.
The figure shows erythrocytes clustered according to their forward scatter (FSC) and sideward scatter (SSC). Intact single cells are gated (R1) for evaluation of their mean fluorescence value and of their fluorescence distribution (FL-1 histogram).

labeled with 9-fluoresceinyl-NeuAc by α2,6-ST were subsequently treated with a high excess of AU-sialidase (100 mU/ml). During 1.5 h at 37°C about 90% of the label was released. After corresponding sialylation with 5-fluoresceinyl-Neu less than 5% of the transferred fluorescence was liberated. Because of sialidase resistance of transferred 5-fluoresceinyl-Neu and superior kinetic data, CMP-5-fluoresceinyl-Neu was preferred as donor substrate for standard labeling procedures.

Optimized conditions for the sialyltransferase-mediated surface labeling with regard to the concentration of enzyme, donor substrate and cell number were determined by flow cytometry. Standard labeling procedure was performed with 40-60 mU/ml sialyltransferase. Dependence on the acceptor concentration was studied by variation of the cell number from 0.3 to 1.5×10^9 per ml assay. Fluorescence incorporated per single cell after 2 h incubation did not depend on the cell concentration. This result assured that sialylation reaction was performed far below acceptor saturation. Concentrations of fluorescent CMP-glycoside above 50 μM did not give a further rise in reaction rate in accordance to the low donor K_M values *(21,22)*.

Table 1: Maximum incorporation of 5-fluoresceinyl-Neu during enzymatic fluorescence labeling mediated by α2,3- and α2,6-sialyltransferase. Cord and adult erythrocytes (native and pretreated by *Arthrobacter ureafaciens* sialidase [=asialo]) were employed. Values were determined by flow cytometry as mean fluorescence and subsequently converted into pmol per 10^6 erythrocytes as described. Given in brackets: Picomol transferred 5-fluoresceinyl-Neu per mg cellular protein.

	native				*asialo*			
	cord		*adult*		*cord*		*adult*	
α2,3-sialyltransferase	*0.6*	*[15]*	*0.8*	*[25]*	*2.5*	*[63]*	*1.8*	*[56]*
α2,6-sialyltransferase	*0.8*	*[20]*	*1.0*	*[31]*	*2.0*	*[50]*	*2.0*	*[62]*
α2,3- and α2,6-sialyltransferase simultaneously	-		*1.2*	*[38]*	-		*2.2*	*[68]*

Sialylation state of lactosaminyl sequences

Expression and sialylation state of lactosaminyl glycans during erythrocyte maturation was investigated by comparison of cord and adult cells. Time course for fluorescence labeling of untreated cord erythrocytes by α2,6-ST determined by flow cytometry (not shown) closely resembled that of adult erythrocytes (Figure 1a). Mean fluorescence values per single cord erythrocyte were constantly 20% lower compared to a mature cell. Maximum values of cytometric mean fluorescence were calculated in terms of incorporated 5-fluoresceinyl-Neu per 10^6 erythrocytes as well as per mg protein (Table 1) (protein content of cord cells is higher than of adult cells).

Following global desialylation of erythrocytes by AU-sialidase, subsequent incorporation of fluorescent sialic acids was used to determine the sialylation extent of surface lactosaminyl glycans. Resialylation kinetics of desialylated cells by α2,6-ST were identical for cord (not shown) and adult erythrocytes (Figure 1a). After 6 h incubation, saturation values of 2.0 pmol per 10^6 cells were achieved indicating an identical extent of accessible acceptor glycans on both cell types (Table 1). The mean sialylation degree of Galβ1,4GlcNAc sequences amounted to only about 50% (fluorescence value of desialylated- compared to that of native cells in Table 1) .

Fluorescence labeling by α2,3-sialyltransferase

Cell surface exposed glycans were further examined by employing a recombinant Galβ1,3(4)GlcNAc α2,3-ST. Both, α2,3-ST and α2,6-ST are introducing sialic acids to

Galβ1,4GlcNAc-, the α2,3-ST additionally to Galβ1,3GlcNAc sequences *(19,25)*. Contribution of the latter sequence was determined by specific enzymatic pretreatment. After selective cleavage of β1,4-linked galactose by the β-galactosidase from *S. pneumoniae* (23), only 20% of surface acceptor sites remained accessible for a fluorescence labeling by α2,3-ST or α2,6-ST in comparison to untreated cells. Surface labeling by α2,3-ST therefore primarily involved Galβ1,4GlcNAc sequences.

Reaction rates of α2,3-ST with fluorescent donors were generally lower compared to that of α2,6-ST. Thus, sialylation in presence of CMP-5-fluoresceinyl-Neu and α2,3-ST did not proceed to saturating level after 9 h at 37°C (Figure 2). To achieve saturation, erythrocytes were first sialylated for 16 hours at 8°C, then a standard sialylation at 37°C for 9 h followed in which surface labeling reached saturation (Table 1). Compared to α2,6-ST, corresponding experiments with α2,3-ST revealed completely different labeling kinetics. During 6 h incubation at 37°C, incorporation of 5-fluoresceinyl-Neu to adult erythrocytes proceeded linear with time whilst labeling of cord cells was approaching saturation (Figure 2). These kinetic differences point to structural variations of the respective acceptor glycans on both cell types.

To determine the sialylation degree, fluorescence incorporation by α2,3-ST in cord and adult erythrocytes was also measured after predesialylation by AU-sialidase. For desialyated adult erythrocytes a maximum value of 1.8 pmol 5-fluoresceinyl-Neu per 10^6 cells was reached after 9 h at 37°C. Cord erythrocytes were first sialylated for 16 h at 8°C followed by a standard sialylation of 9 h at 37°C, which approximated to the

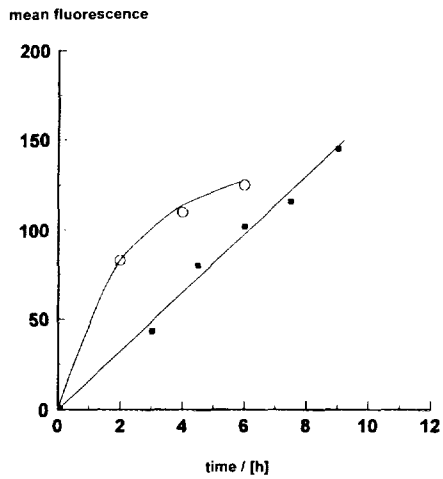


Figure 2. Time course for labelling of adult (■) and cord (o) erythrocytes by recombinant Galβ1,4(3)GlcNAc α2,3-ST (60mU/ml) employing CMP-5-fluoresceinyl-Neu (50μM) as determined by flow cytometry.

saturation value of about 2.5 pmol per 10^6 cells (Table 1). This transfer of fluorescent sialic acid was higher than the value obtained with α2,6-ST (Table 1).

The significant differences in the amount of acceptor sites detected by each of the two sialyltransferases (Table 1) pointed against a complete overlapping in specificity towards Galβ1,4GlcNAc sequences. In accordance, simultaneous sialylation in presence of α2,3-ST and α2,6-ST achieved slightly higher final fluorescence values with native and desialylated adult erythrocytes than in presence of only α2,6-ST (Table 1).

Detection of polylactosaminyl sequences

Further structural characterization of glycoconjugates carrying the fluorescent sialic acid was achieved by treatment of labelled cells with endoglycosidase and endoprotease. Endo-β-galactosidase specifically cleaves internal Galβ1,4GlcNAc repeats within polylactosaminyl glycans (26) which are found in band 3 and band 4.5 *(27,28)*. Complex-type glycans of glycophorins remain resistant to this enzymatic treatment *(29)*. After a saturated incorporation of 5-fluoresceinyl-Neu by α2,6-ST, cord and adult erythrocytes were subsequently treated by endo-β-galactosidase. Following up the time course by flow cytometry, the final fluorescence release measured after 1.5 h with cord and adult erythrocytes was 45% and 30%, respectively. After corresponding sialylation by α2,3-ST surface fluorescence of both cell types was reduced by 65%.

Mild trypsin treatment of intact erythrocytes removes the complex type glycans of glycophorin A, whereas the glycoproteins carrying polylactosaminyl sequences remain unaffected *(29)*. Trypsination of labeled adult erythrocytes yielded 20% and 50% release of the fluorescence incorporated by α2,3-ST and α2,6-ST, respectively.

These results point to marked differences in fine specificity of both enzymes. The rat liver α2,6-ST transferred the fluorescent sialic acid to complex-type- and, with less efficiency, to polylactosaminyl glycans, whereas the recombinant α2,3-ST predominantly labeled polylactosaminyl sequences.

DISCUSSION

We here describe a novel approach for single cell analysis of distinct glycan sequences exposed at the surface of intact cells. This enzymatic analysis was first described employing radiolabeled CMP-NeuAc *(15-18)*. The new procedure is based on the introduction of fluorescent sialic acids to particular glycan sequences according to the acceptor specificitiy of an exogenously applied sialyltransferase (ST) and is suited for evaluation of the labeled intact cells by flow cytometry.

Two synthetic CMP-glycosides substituted by a fluoresceinyl-residue at position C-5 or C-9 of the sialic acid moiety served as fluorescent donor substrates. CMP-5-fluoresceinyl-Neu was preferably employed since (i) this compound yields superior kinetic properties and (ii) 5-fluoresceinyl-Neu transferred is resistant towards sialidases (21). A rat liver α2,6-sialyltransferase strictly specific for the Galβ1,4GlcNAc sequence, and a recombinant α2,3-sialyltransferase specific for the Galβ1,4GlcNAc- as well as for the Galβ1,3GlcNAc-sequence served as tools for tagging of defined glycans *(19,25)*.

The sialyltransferase mediated analysis described is superior to respective lectin-dependent- and chemical methods for the following reasons: compared to lectin staining, [i] the enzyme is strictly specific for a certain glycan acceptor sequence; [ii] the label is covalently linked thus allowing the product characterization on intact or lysed cells by endoglycosidases or endoproteases, [iii] and the absolute amount of fluorescent sialic acid can easily be quantified using the chromatographic system developed previously *(22)*. [iv] In contrast to lectin-staining, the sialyltransferase-mediated labeling does not induce cell agglutination and can thus proceed to a saturating level. [v] The respective chemical procedures for labeling of sialic acid or galactose residues are suffering predominantly from a loss of selectivity towards the penultimate glycan sequence *(30,31)*.

The enzymatic transfer of fluorescent sialic acids to surface glycans, evaluated by flow cytometry, permits very sensitive quantification of surface bound fluorescence at the level of a single cell. Electronical gating of the data according to the light scattering signals allows exclusion of damaged cells and cell debris. The electronical gating also facilitates cell differentiation, e.g. erythrocytes, platelets, monocytes or granulocytes, without special separation procedures. Analytical data restricted to viable cells are more difficult to obtain by the corresponding radiometric procedure. By measurement of 20000 single cells a valid mean value for the labelling extent is calculated. In contrast to the sialyltransferase-mediated radiolabeling, the distribution of incorporated fluorescent sialic acid within the cell population can be monitored in detail (Histogram of Fig. 1b), and the fluorescence assay can be easily performed at donor saturation due to the low K_M value of the fluorescent CMP-glycosides (21,22).

The flow cytometric glycan analysis was applied here to compare accessible lactosaminyl glycans during maturation of human erythrocytes. Cord and adult cells were used, since the structures of their membrane glycans have already been elucidated. Thus, the effect of endoglycosidase and endoproteases can be correlated to certain glycans *(26-29)*. The results obtained by the novel method can be compared to previous data assessed by differring analytical techniques. In contrast to these procedures, the sialyltransferase-mediated fluorescence labeling focuses on sterically

accessible glycans of live cells, since the enzymatic labeling of lysed- was 3fold higher compared to intact erythrocytes (not shown).

The most striking difference among the N-linked surface glycans during maturation of erythrocytes is the increase in branching of polylactosaminyl sequences *(27,28)*. In spite of this, the label transferred to desialylated cord and adult cells by α2,6-ST was not different (Table 1). Expression of acceptor glycans for α2,3-ST on desialylated cord cells was higher than on adult (Table 1), the increase in branching affected only sialyltransfer by this enzyme. At both maturation stages high incorporation of fluorescent sialic acid was yielded to untreated erythrocytes (Table 1). This high expression of non-sialylated Galβ1,4GlcNAc sequences may represent recognition sites for lectin receptors on liver-, spleen-, and RES cells, which for example facilitate the removal of aged erythrocytes from circulation *(32)*. The broad distribution of surface fluorescence within the cell population (FL-1 histogram, Fig. 1b) presumably reflects at least in part an age dependent loss of sialic acid.

It has to be considered that for reasons of branch and fine specificity *(33)* a sialyltransferase may not react with a particular glycoconjugate, though the respective oligosaccharide chain fits to the appropriate basic acceptor sequence. Special variations like fucosylated or highly branched lactosaminyl sequences are resistant towards enzymatic sialyltransfer. Further, the subsequent treatment of labeled erythrocytes by endo-β-galactosidase or trypsin revealed a significant difference in fine specificity of α2,3-ST and α2,6-ST, and indicated that not all types of lactosaminyl glycans were labeled with equal efficiency by either enzyme. The rat liver α2,6-ST defines expression and sialylation state of complex-type- and polylactosaminyl-, the recombinant α2,3-ST preferentially of polylactosaminyl glycans. In conclusion, advantage can be taken from these differences to distinguish the cell specific types of acceptor glycans.

ACKNOWLEDGMENTS

The authors are indepted to Dr. J. C. Paulson (CYTEL Incorp.) for generously providing the recombinant α2,3-sialyltransferase, and to J.M. Krause for expert technical assistance. The work was supported by grant Gr 990/1-2 of the Deutsche Forschungsgemeinschaft (DFG), by the Mörsel Foundation (Medizinische Fakultät, Universität Heidelberg), and by Boehringer Mannheim (Dr. Th. Priester; Biochemica).

REFERENCES

1. Passaniti, A., and Hart, G.W. (1988) *J. Biol. Chem.* **263,** 7591-7603
2. Crocker, R., Kelm, S., Dubois, C., Martin, B., Mc William, A.S., Shatton, D.M., Paulson, J.C., and Gordon, S. (1991) *EMBO J.* **10,** 1661-1669

3. Feizi,T. (1991) *TIBS* **16,** 84-86

4. Varki, A. (1993) *Glycobiology* **3,** 97-130

5. Schwartz-Albiez, R., Moldenhauer, G., Kuipers J., Möller, P., Funderud, S., and Bast, B. (1994) *In* Leukocyte Typing V (Schlossmann, S. Ed.), Univ. Press, Oxford, in press

6. Kelm, S., Pelz, A., Schauer, R., Filbin, M.T., Tang, S., Bellard, M.E., Schnaar, R.L., Mahoney, J.A., Hartnell, A., Bradfield, P., and Crocker, P.R. (1994) Curr. Biol. **4,** 965-973

7. Powell, L.D., and Varki, A. (1994) *J. Biol Chem.* **269,** 10628-10636

8. Springer, T.A. (1994) *Cell* **76,** 301-314

9. Varki, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7390-7397

10. Ashwell G., and Harford, J. (1982) *Annu. Rev. Biochem.***51,** 531-534

11. Hughes, C. (1994) *Glycobiol.* **4,** 5-12

12. Feizi,T. (1985) *Nature* **314,** 53-57

13. Roelcke, D., Hengge U., and Kirschfink, M. (1990) *Vox Sang.* **59,** 235-239

14. Beppu, M., Mizukami, A., Ando, K., and Kitagawa, K. (1992) *J. Biol. Chem.* **267,** 14691-14696

15. Whiteheart, S.W., and Hart, G.W. (1987) *Anal. Biochem.* **163,** 123-135

16. Passaniti, A., and Hart, G.W. (1987) J. Biol. Chem. 263, 7591-7603

17. Whiteheart, S.W., Passaniti, A., Reichner, J.S., Holt, D.J., Haltiwanger R.S., and Hart, G.W. (1989) *Methods Enzymol.* **179,** 82-95

18. Haltiwanger, R.S,. and Hart, G.W. (1993) Methods Mol. Biol. 14, 175-187

19. Wen, D.X. Livingston, B.D., Medzihradszky, K.F., Kelm, S., Burlingame, A.L.,and Paulson, J.C. (1992) *J. Biol. Chem.* **267,** 21011-21019

20. Groß, H.J., and Brossmer, R. (1988) *Eur. J. Biochem.* **177,** 583-589

21. Brossmer, R., and Groß H.J. (1994) *Methods Enzymol.* **247 B,** 177-192

22. Groß, H.J., Sticher, U., and Brossmer, R. (1990), *Anal. Biochem.* **186,** 127-134

23. Sadler, J.E., Paulson, J.C., and Hill, R.L. (1979) *J. Biol. Chem.* **254,** 2112-2119

24. Becker, K., Krebs, B., and Schirmer, R.H. (1991) *Internat. J. Vit. Nutr. Res.* **61,** 180-187

25. Weinstein, J. , Souza-e-Silva, U., and Paulson, J.C. (1982) *J. Biol. Chem.* **257,** 13845-13853

26. Scudder, P., Uemura, K., Dolby, J., Fukuda, M., and Feizi, T. (1983) *Biochem. J.* ***213,*** 485-592

27. Viitala, J., and Järnefelt, J. (1985) *TIBS* **10,** 392-395

28. Fukuda, M. (1985) *Biochim. Biophys. Acta* **780,** 119-150

29. Dzandu, J.K., Deh, M.E., and Wise, G.E. (1985) *Biochem. Biophys. Res. Comm.* **126,** 50-58

30. Spiegel, S. (1987) *Methods Enzymol.* **138,** 313-318

31. Wilchek, M., and Bayer, E.A. (1987) *Methods Enzymol.* **138,** 429-442

32. Reutter, W., Köttgen, E., Bauer, C., and Gerok, W. (1982) *In* Sialic Acids Chemistry, Metabolism and Function (Schauer, R., Ed.); pp. 265-305, Springer, Wien

33. Joziasse, D.H, Schiphorst W.E.C.M., Van den Eijnden, D.H., Van Kuik, J.A., Van Halbeek, H., and Vliegenhart J.F.G (1987), *J. Biol. Chem.* **262,** 2025-2033