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Species-specific expression of sialosyl-Le^x on polymorphonuclear leukocytes (PMN), in relation to selectin-dependent PMN responses

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Sialosyl-Le^x (SLe^x) and its positional isomer sialosyl-Le^a are the epitopes recognized by the lectin domain of E- and P-selectins. Expression of SLe^x in polymorphonuclear leukocytes (PMN) plays an important role in recruitment of these cells at sites of inflammation through activation of selectins. We studied expression of SLe^x in PMN of seven mammalian species in comparison with that in humans. Only PMN of humans (no other species) expressed SLe^x or other lacto-series epitopes such as Le^x or Le^y. The observed absence of these epitopes in rat PMN seems inconsistent with recent reports that the lung inflammation process in a rat model is inhibited by perfusion of SLe^x oligosaccharide (Mulligan MS, *et al.* (1993a) *Nature* **364**:149; (1993b) *J Exp Med* **178**:623). Rat selectins may be able to recognize SLe^x, even though this epitope is absent in rat PMN.

Keywords: PMN, selectin, SLe^x, SLe^a, adhesion molecule

Abbreviations: FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PMN, polymorphonuclear leukocytes; SLe^a, sialosyl-Le^a antigen; SLe^x, sialosyl-Le^x antigen.

Introduction

An important factor in inflammatory responses is the recruitment of polymorphonuclear leukocytes (PMN[1]) (primarily neutrophils) into tissues through a multi-step process. This process involves sequential engagement of adhesion molecules, particularly E- and P-selectins, which are claimed to be reactive with sialosyl-Le^x (SLe^x) or its positional isomer sialosyl-Le^a (SLe^a) [1–5]. Selectin-mediated adhesion is followed by adhesion mediated by $\beta 2$ integrins, which are reactive with endothelial intercellular adhesion molecules (ICAMs) [6, 7]. The rabbit ear reperfusion injury model has been used to test effectiveness in such 'anti-adhesion therapy' of mAbs directed to P-selectin [8] as well as to CD18, the $\beta 2$ integrin receptor present at the PMN surface [9]. Since selectin-dependent rolling of PMN on endothelial cells occurs prior to $\beta 2$ integrin/ICAM adhesion [7], the effect of SLe^x oligosaccharides as possible inhibitors of inflammatory response has been intensively studied in a few experimental models. In a rat model of acute lung injury induced by i.v. infusion of cobra venom factor, PMN-dependent damage of lung tissue, caused by O_2^- production, is mediated by platelet activation and P-selectin expression [10, 11], and the inflammatory process was inhibited by infusion of SLe^x oligosaccharide or its derivatives [12], which are claimed to be ligands of P-selectin in humans. In another acute inflammation model, rat lung injury is caused by deposition of IgG immune complex, and E-selectin plays a major role in PMN-dependent tissue damage. In this case, however, the inhibitory effect of SLe^X was weak compared to that of infusion of Chinese Hamster Ovary cells transfected with α1,3/4 fucosyltransferase, which produced a 40–70% reduction of inflammatory response index [13]. These results suggest that P- or E-selectin in rat recognizes SLe^x, which is generally assumed to be expressed on rat PMN.

We now report systematic studies on expression of various lacto-series type 1 and type 2 chain epitopes on PMN from humans and various experimental mammalian species. Surprisingly, SLe^x and other type 2 chain structures were expressed only on human PMN. Human PMN did not express type 1 chain. None of the seven other species examined expressed SLe^x, SLe^a, or any other lacto-series type 1 or type 2 epitope.

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Table 1. Carbohydrate structures defined by various mAbs used in this study.

mAb name	*Structure defined*	*Isotype*	*Reference*
1B2	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → R	IgM	20
AH6	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → R 2 3 ↑ ↑ Fucα1 Fucα1	IgM	21
CA3F4	Galβ1 → 3GlcNAcβ1 → 3Galβ1 → R 4 ↑ Fucα1	IgM	22
FH2	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → R 3 ↑ Fucα1	IgM	23
SH1	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → R 3 ↑ Fucα1	IgG_3	24
FH6	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → R 3 3 3 ↑ ↑ ↑ NeuAcα2 Fucα1 Fucα1	IgM	25
SNH4	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → R 3 3 ↑ ↑ NeuAcα2 Fucα1	IgG_3	1, 26[a]
N19-9	Galβ1 → 3GlcNAcβ1 → 3Galβ1 → R 3 4 ↑ ↑ NeuAcα2 Fucα1	IgG_1	27

[a] Detailed production methods and properties of this mAb have not been published.

Materials and methods

Antibodies used, and structures defined

The mAbs used for determination of various carbohydrate epitopes, and literature citations, are listed in Table 1. These mAbs were used for indirect flow cytometry, i.e. treatment of cells with culture supernatant from hybridoma containing 10–20 µg of Ig per ml, followed by treatment with fluorescence-labelled anti-mouse goat antibody. MAbs FH6, N19-9, and SH1 were purified, labelled directly with FITC, and used for direct staining followed by flow cytometry. For the indirect method, 2×10^5 cells were incubated for 1 h on ice with supernatant of each mAb culture, which contained 5–10 µg Ig per ml of RPMI medium. Cells were washed three times with PBS containing 1% BSA and 0.1% sodium azide ('washing buffer'), incubated for 1 h on ice with 1:40 diluted FITC-conjugated goat anti-mouse IgM/IgG, washed with washing buffer, and analysed on an EPICS flow cytometer (Coulter Corp., Hialeah, FL, USA).

For the direct method, 2×10^5 cells were incubated with mouse IgG for 45 min, washed with washing buffer, incubated for 1 h on ice in a medium containing 10 µg ml^{-1} of mAb directly conjugated with FITC, washed three times with washing buffer and analysed on a flow cytometer.

Isolation of PMN from blood and peritoneal effusion

Blood from healthy human subjects or from various experimental mammalian species (Table 2) was drawn and mixed with preservative-free heparin, then mixed with 6% dextran in saline (ratio 2:1), and allowed to stand for 1 h at room temp. The upper layer was collected, layered over the same volume of Ficoll-Paque™ (Pharmacia LKB, Uppsala, Sweden) (for blood of humans, baboons, and macaques) or Nyco-Prep™ 1.077 (Nycomed, Oslo, Norway) (for blood of non-primate species), and centrifuged for 20 min at 600 × **g**. Ficoll-Paque and Nyco-Prep exhibit a small osmolarity difference (according to the instructions of Nycomed), but do not change antibody-binding properties of PMN. Pellets were collected, and erythrocytes were lysed by the addition of 1 ml of ice-cold water for 30 s followed by 1 ml of 1.8% saline. Cells were washed twice with PBS. Purity (determined by morphological examination of PMN smear on glass plate stained by Giemsa's dye) and viability (determined by Trypan blue exclusion test) of PMN were

Table 2. Indirect immunostaining of mammalian PMN by various antibodies (% positive).

	Mouse IgG	*AH6*	*N19-9*	*1B2*	*SNH4*	*FH6*	*CA3F4*	*SH1*	*FH2*
Human	0.7	95.9	0.1	22.8	97.4	99.6	0.1	86.0	63.8
Baboon	1.4	0.6	0.5	0.7	0.5	0.6	0.4	0.4	0.8
Macaque	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pig	0.9	0.0	0.0	1.1	1.1	0.5	0.1	0.0	0.0
Rabbit (New Zealand White)	0.2	0.2	0.1	0.0	1.4	8.9	0.0	0.0	0.0
Rat	3.4	2.5	3.7	3.2	2.9	2.9	3.3	2.7	2.9
Guinea pig (Hartley)	3.8	4.3	3.2	3.4	6.3	7.9	3.1	3.6	3.3
Peritoneal effusion	2.2	2.1	2.1	1.9	3.5	3.7	2.5	2.5	2.2
Hamster (Syrian)	2.9	2.7	14.8[a]	3.6	1.7	2.3	2.8	2.1	3.6
Peritoneal effusion	1.5	0.3	0.6	0.3	0.5	0.5	0.4	0.4	0.7

All PMN were taken from peripheral blood except for two peritoneal effusion samples as indicated. Values for baboon, macaque, and pig represent a single sample; all other values represent mean of two samples.
[a] This anomalously high value was not reproduced in direct binding assay (Table 3).

consistently >95%. Rat PMN were prepared from Wistar strain and Long-Evans strain rats. The latter was the same strain used in the previous studies by Mulligan *et al.* [12, 13].

Peritoneal effusion was prepared by i.p. injection of 10 ml of sterile 2% casein solution in PBS into guinea pig (20 ml), rat (10 ml), or hamster (10 ml). After 16 h, the peritoneal cavity was washed by PBS infusion [14]. Cells present in the effusion were mostly (>95%) PMN, and were collected by centrifugation.

Selectin-dependent adhesion of rat vs. *human PMN*

Soluble fusion proteins of human E- or P-selectin were prepared from Chinese Hamster Ovary DG44 cells (obtained from Dr L. A. Chasin, Columbia University, NY, USA) co-transfected with pCDM8 containing E- or P-selectin and human IgG_1 Fc portion [15, 16] (donated by Dr Brian Seed, Massachusetts General Hospital, Boston, MA, USA) and psV2/dhfr (ATCC, Rockville, MD, USA). Ninety-six-well flat-bottom microtitre plates (Falcon, Becton-Dickinson, Lincoln Park, NJ, USA) were coated with goat anti-human IgG antibody (50 µl of 5 µg ml^{-1} solution per well). Wells were washed three times with PBS, blocked with 5% BSA in PBS, coated with 100 µl of selectin fusion protein (culture supernatant) or human IgG_1 in PBS for 2 h at room temp, and washed three times with PBS. 1×10^5 cells labelled with 2′, 7′-bis(carboxyethyl)-5(6′)-carboxyfluorescein (Molecular Probe, Eugene, OR, USA) were added to each well and allowed to bind for 20 min at room temperature. After unbound cells were washed out, bound cells were lysed with 1% Triton X-100 solution, and the released fluorescence was determined using a Perkin–Elmer LS-2B Filter Fluorimeter.

Table 3. Direct immunostaining of mammalian PMN and HL60 cells by various antibodies (% positive).

	Mouse IgG	*Mouse IgM*	*FH6*	*N19-9*	*SH1*
HL60 cells	0.1	1.2	95.5	0.0	67.0
Human PMN	0.4	0.2	99.9	0.4	88.0
Rat PMN[a]	0.5	0.1	1.9	0.3	2.8
Hamster PMN	1.9	0.2	3.5	3.4	3.3

Values for rat represent mean of four samples; all other values represent mean of two samples.
[a] Values did not differ between Wistar *vs* Long-Evans rat strains.

Results and discussion

The expression of carbohydrate antigens on PMN of humans and other mammalian species, as determined by the indirect method, is summarized in Table 2. These results were confirmed by flow cytometry using purified mAbs directly conjugated to FITC (Table 3). Only human PMN expressed lacto-series type 2 chain structures such as Le^y (defined by mAb AH6), Le^x (defined by SH1 and FH2), and SLe^x (defined by SNH4 and FH6). Human PMN did not express type 1 chain structures such as Le^a (defined by CA3F4) or SLe^a (defined by N19-9). None of the experimental animals examined, even primates such as the baboon or rhesus macaque, expressed either lacto-series type 1 or type 2 chain structures on their PMN. Since rats are frequently-used experimental models (see Introduction), we carefully checked our results using a direct antibody-binding assay. Multiple experiments with rat PMN were all negative in binding with purified FH6, N19-9, or SH1. Rat PMN in peritoneal effusion also gave negative results in direct binding assay with all types of mAb. Human PMN and human promyelogenous leukemia HL60 cells were positive with FH6 and SH1, confirming the results with indirect binding assay. Some representative flow cytometric patterns are shown in Fig. 1. Neither mouse IgG nor mouse IgM (used as controls) showed any staining.

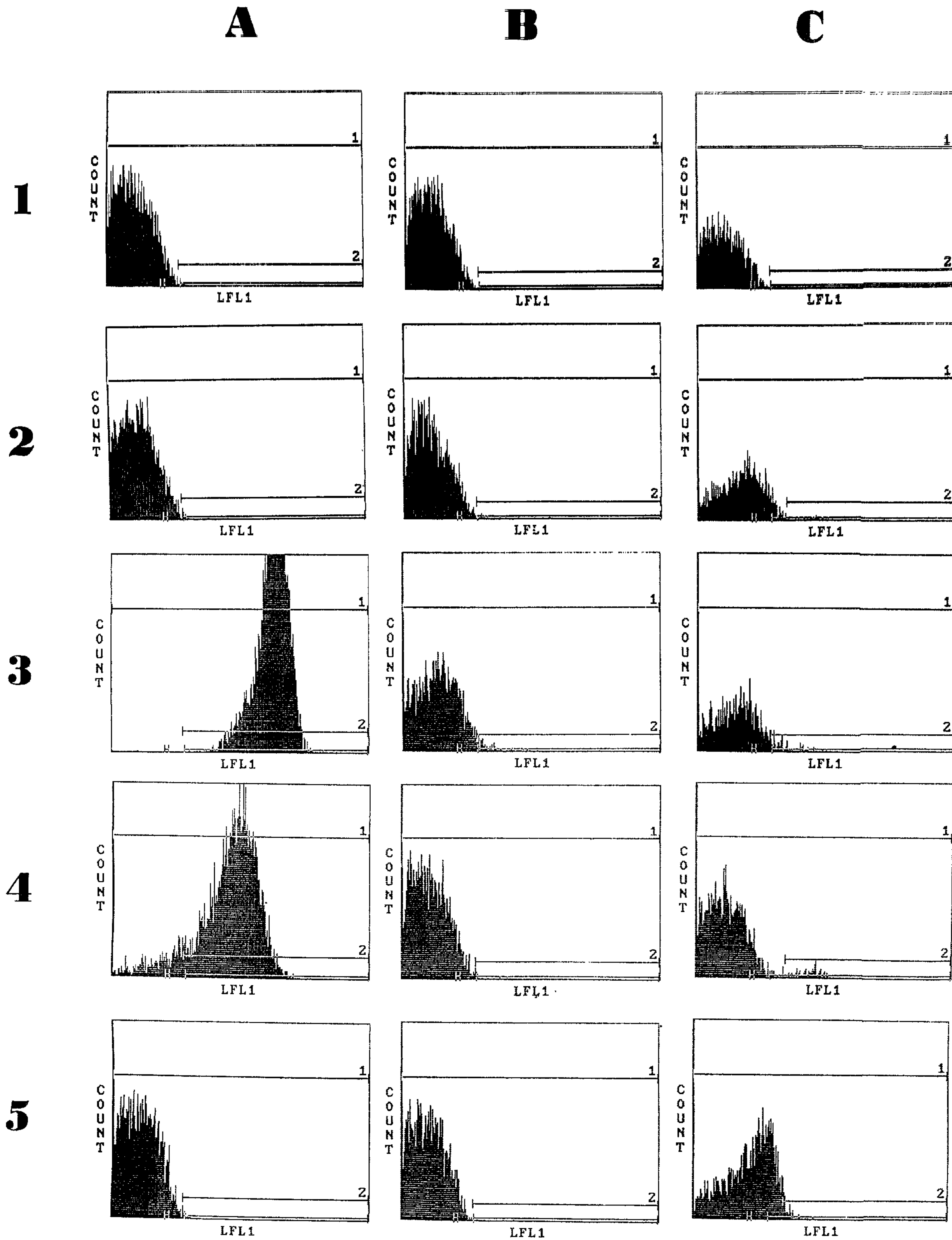

Figure 1. Typical flow cytometric patterns of PMN after direct binding with various antibodies. Experimental conditions are described in the text. Column A: human PMN. Column B: rat (Long-Evans strain) PMN. Column C: hamster PMN. Row 1: control mouse IgM antibody. Row 2: control mouse IgG antibody. Row 3: mAb FH6. Row 4: mAb SH1. Row 5: mAb N19-9.

Table 4. Cell binding assay using 96-well microtitre plates coated with soluble fusion proteins.

	Specific binding (%)	
	Human E-selectin	*Human P-selectin*
HL60 cells	38.8 ± 1.6	39.1 ± 2.2
Human PMN	27.5 ± 1.2	19.2 ± 1.1
Rat PMN[a]	3.0 ± 0.2	<1
Rabbit PMN	4.0 ± 1.0	<1

Values represent number of cells bound to wells coated with selectin-$hIgG_1$ fusion protein (background binding values to $hIgG_1$ control were subtracted), expressed as a percentage of total number of cells added to each well. Mean ± SD of triplicate determination. All binding values were <1% in the presence of ethylenediaminetetraacetic acid (EDTA), which abolishes selectin activity.

[a] Data from Wistar strain rats. Long-Evans strain rats showed similar binding values.

In adhesion assay using E- or P-selectin fusion proteins with human Ig Fc region, only HL60 cells and human PMN showed positive binding. In contrast, adhesion of rat PMN to E- or P-selectin-coated plates was negligible (Table 4).

We have confirmed the expression of SLe^x and Le^x, and absence of type 1 chain SLe^a and Le^a, in human PMN and HL60 cells, as previously reported by us and others [17,18]. Our results indicate that PMN expression of SLe^x, Le^x, and Le^y is highly species-specific to humans. Therefore, anti-adhesive effects of SLe^x oligosaccharide or its derivatives using animal models of inflammation requires careful evaluation. In previous experiments based on a rat lung injury model [12, 13], SLe^x oligosaccharide or derivatives were administered in an attempt to inhibit inflammatory response caused by PMN recruitment. Some results were impressively positive, i.e. SLe^x oligosaccharide had an inhibitory effect whereas the control oligosaccharide sialosyl type 2 chain (NeuAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 4GlcNAc) had no effect. However, the basis for these observations is unknown since rat PMN obviously do not express SLe^x nor SLe^a. One possibility is that binding specificity of E- and P-selectins is maintained across various species. Perhaps rat selectins are capable of binding to SLe^x, and this binding is inhibitable by SLe^x oligosaccharides and derivatives even though rat PMN do not express SLe^x.

Recognition of PMN by selectins is believed to be a common physiological defense mechanism in humans and other mammals. In humans, the recognition molecule is thought to be SLe^x or its analogues expressed on PMN and bound by E- and P-selectins [1, 2], although the true naturally-occurring epitope recognized under dynamic flow conditions remains unclear [3, 19]. The absence of SLe^x or related structures (Le^x, Le^y, Le^a) in PMN of seven non-human mammalian species is surprising, since all these species are characterized by selectin expression and recognition of PMN by selectins. Since immobilized human P- and E-selectins do not bind to rat or rabbit PMN, we assume that other specific (but so far unidentified) carbohydrate structures are involved in selectin-PMN recognition in rat, rabbit, and other non-human mammalian species. Nevertheless, rat selectins are probably still capable of binding SLe^x, as suggested by the studies by Mulligan *et al.* [12, 13] on rat inflammatory response. Perhaps, although SLe^x expression on PMN is restricted to humans, there is little variation in binding specificity among selectins of different mammalian species.

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References

1. Phillips ML, Nudelman ED, Gaeta FCA, Perez M, Singhal AK, Hakomori S, Paulson JC (1990) *Science* **250**:1130–32.
2. Polley MJ, Phillips ML, Wayner EA, Nudelman ED, Singhal AK, Hakomori S, Paulson JC (1991) *Proc Natl Acad Sci USA* **88**:6224–28.
3. Handa K, Nudelman ED, Stroud MR, Shiozawa T, Hakomori S (1991) *Biochem Biophys Res Commun* **181**:1223–30.
4. Takada A, Ohmori K, Takahashi N, Tsuyuoka K, Yago A, Zenita K, Hasegawa A, Kannagi R (1991) *Biochem Biophys Res Commun* **179**:713–19.
5. Berg EL, Robinson MK, Mansson O, Butcher EC, Magnani JL (1991) *J Biol Chem* **266**:14869–72.
6. Butcher EC (1991) *Cell* **67**:1033–36.
7. Lawrence MB, Springer TA (1991) *Cell* **65**:859–73.
8. Winn RK, Liggitt D, Vedder NB, Paulson JC, Harlan JM (1993) *J Clin Invest* **92**:2042–47.
9. Vedder NB, Winn RK, Rice CL, Chi EY, Arfors KE, Harlan JM (1990) *Proc Natl Acad Sci USA* **87**:2643–46.
10. Mulligan MS, Polley MJ, Bayer RJ, Nunn MF, Paulson JC, Ward PA (1992) *J Clin Invest* **90**:1600–7.
11. Larsen E, Celi A, Gilbert GF, Furie BC, Erben JK, Bonfanti R, Wagner DD, Furie B (1989) *Cell* **59**:305–12.
12. Mulligan MS, Paulson JC, DeFrees S, Zheng Z-L, Lowe JB, Ward PA (1993) *Nature* **364**:149–51.
13. Mulligan MS, Lowe JB, Larsen RD, Paulson JC, Zheng Z-L, DeFrees S, Maemura K, Fukuda M, Ward PA (1993) *J Exp Med* **178**:623–31
14. Kohama Y, Kayamori Y, Katayama Y, Teramoto T, Murayama N, Tsujikawa K, Okabe M, Ohtani T, Matsukura T, Mimura T (1992) *Chem Pharm Bull* **40**:414–18.

15. Walz G, Aruffo A, Kolanus W, Bevilacqua MP, Seed B (1990) *Science* **250**:1132–35.
16. Aruffo A, Kolanus W, Walz G, Freedman P, Seed B (1991) *Cell* **67**:35–44.
17. Symington FW, Hedges DL, Hakomori S (1985) *J Immunol* **134**:2498–506.
18. Fukuda M, Spooncer E, Oates JE, Dell A, Klock JC (1984) *J. Biol Chem* **259**:10925–35.
19. Kojima N, Handa K, Newman W, Hakomori S (1992) *Biochem Biophys Res Commun* **189**:1686–94.
20. Young WW Jr, Portoukalian J, Hakomori S (1981) *J Biol Chem* **256**:10967–72.
21. Abe K, McKibbin JM, Hakomori S (1983) *J Biol Chem* **258**:11793–97.
22. Young WW Jr, Johnson HS, Tamura Y, Karlsson K-A, Larson G, Parker JMR, Khare DP, Spohr U, Baker DA, Hindsgaul O, Lemieux RU (1983) *J Biol Chem* **258**:4890–94.
23. Fukushi Y, Hakomori S, Nudelman ED, Cochran N (1984) *J Biol Chem* **259**:4681–85.
24. Singhal AK, Singhal MC, Nudelman ED, Hakomori S, Balint JP, Grant CK, Snyder HW Jr (1987) *Cancer Res* **47**:5566–71.
25. Fukushi Y. Nudelman ED, Levery SB, Rauvala H, Hakomori S (1984) *J Biol Chem* **259**:10511–17.
26. Muroi K, Suda T, Nojiri H, Ema H, Amemiya Y, Miura Y, Nakauchi H, Singhal AK, Hakomori S (1992) *Blood* **79**:713–19.
27. Magnani JL, Nilsson B, Brockhaus M, Zopf D, Steplewski Z, Koprowski H, Ginsburg V (1982) *J Biol Chem* **257**:14365–69.