THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 49, Issue of December 6, pp. 31526–31532, 1996
Printed in U.S.A.

Sialic Acid 9-*O*-Acetylation on Murine Erythroleukemia Cells Affects Complement Activation, Binding to I-type Lectins, and Tissue Homing*

(Received for publication, September 3, 1996, and in revised form, September 27, 1996)

Wei-Xing Shi, Roger Chammas‡, Nissi M. Varki, Leland Powell, and Ajit Varki§

From the Glycobiology Program, UCSD Cancer Center, the Division of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California 92093

***O*-Acetylation of the 9-hydroxyl group of sialic acids has been suggested to modify various recognition phenomena involving these molecules, but direct proof has been lacking in most situations. In the accompanying paper (Shi, W.-X., Chammas, R., and Varki, A. (1996) *J. Biol. Chem.* 261, 31517–31525), we report that the extent of 9-*O*-acetylation of cell surface sialic acids on murine erythroleukemia (MEL) cells can be modified by various manipulations, including differentiation, nocodazole treatment, and 9-*O*-acetyl esterase treatment. We have used this system to explore the putative roles of 9-*O*-acetylation in modulating alternative pathway complement activation, I-type lectin binding, and tissue homing. MEL cells are shown to be sensitive to lysis *in vitro* by the alternative pathway of human complement. Induced differentiation of the MEL cells causes resistance to lysis, and this correlates directly with extent of decrease in 9-*O*-acetylation. A similar resistance to alternative pathway lysis can be obtained by selective enzymatic removal of 9-*O*-acetyl groups from sialic acids. Conversely, the increase in cell surface 9-*O*-acetylation caused by nocodazole treatment correlates with increased sensitivity to alternative pathway lysis. Thus, a 9-*O*-acetyl group added to the side chain of cell surface sialic acids may abrogate its normal function in restricting alternative pathway activation. Indeed, the binding of human complement factor H, a negative regulator of the alternative pathway, is shown to be blocked by *O*-acetylation of the sialic acids on MEL cells. MEL cells are also shown to have cell surface ligands for the I-type lectins sialoadhesin and CD22. Sialoadhesin (but not CD22) binding is selectively enhanced by differentiation-induced loss of cell surface 9-*O*-acetylation and by direct enzymatic removal of the ester groups. Thus, some sialoadhesin ligands are masked by 9-*O*-acetylation, presumably because the side chain is required for recognition. Since sialoadhesin is expressed on some macrophages *in vivo*, we reasoned that tissue homing of MEL cells might be affected by *O*-acetylation. Indeed, enzymatic removal of cell surface 9-*O*-acetyl groups alters the tissue distribution of intravenously injected cells. In particular, de-*O*-acetylation caused significant increase in homing to the liver and spleen. These data demonstrate that cell surface 9-*O*-acetylation can affect a variety of biological recognition phenomena and provide a system for further exploration of the specific molecular mechanisms involved.**

Sialic acids (Sias)[1] are a family of 9-carbon carboxylated monosaccharides often found as terminal residues of vertebrate oligosaccharides (1). Sialic acid-containing cell surface glycoconjugates (sialoglycoconjugates) are known to influence many biological processes. For example, they may alter the physical properties of the plasma membrane or serve as specific ligands for certain lectins and toxins (1). Sialic acids can also be modified in a variety of ways, giving rise to a family of more than 30 different structures (2, 3) which, in turn, can be presented in a variety of linkages to the underlying sugar chain. These modifications can significantly affect the physicochemical properties of the parent molecule and are therefore predicted to modify their function and/or to create new functions. In mammals, one of the most common modifications of sialic acids is the addition of *O*-acetyl esters to the hydroxyl group at the C-9 position. These esters are well known to affect recognition of Sias by viral hemagglutinins and bacterial sialidases (2, 3) and by the recently described sialic acid-binding mammalian proteins belonging to the I-type lectin family (4–8). Indirect evidence also suggests that they may affect tissue morphogenesis during development (9) and modulate the alternative pathway of complement activation (10).

Murine erythroleukemia (MEL) cells are virus-transformed erythroid precursors that proliferate in culture until they are given a signal for terminal differentiation (11). In the preceding paper (12), we showed that expression of 9-*O*-acetylation on MEL cells is a highly regulated modification, being selectively found on cell surface mucin-type glycoproteins, down-regulated upon cellular differentiation, and affected by the integrity of the microtubular network. We also showed that a recombinant soluble form of the influenza C hemagglutinin esterase (13) allows direct and selective removal of 9-*O*-acetyl esters from the surface of MEL cells. This work has provided a system wherein the functional consequences of altering the level of cell surface 9-*O*-acetylation can be directly examined.

Here we have investigated the influence of modulating the levels of MEL cell 9-*O*-acetylation upon the activation of alter-

* This research was supported by grant RO1-GM32373 (to A. V.) and PO1-CA5869 (to M. Farquhar). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Postdoctoral Fellowship 96/0314-8 from Fundação de Amparo a Pesquisa do Estado de São Paulo, Brasil.

§ To whom correspondence should be addressed. Tel.: 619-534-3296; Fax: 619-534-5611.

[1] The abbreviations used are: Sia, sialic acid; PBS, phosphate-buffered saline; CD22Rg, chimeric protein made of the first three extracellular domains of CD22β, fused to the Fc portion of human IgG_1; CHE-Fc, chimeric protein made of InfCHE (influenza C hemagglutinin esterase with the fusion peptide eliminated by mutation) and the Fc portion of human IgG_1; CHE-FcD, diisopropyl fluorophosphate-treated CHE-Fc (esterase activity irreversibly inactivated); GVB, veronal-buffered saline containing 0.1% gelatin; GVB-Mg-EGTA, GVB containing 2 mM Mg^{2+} and 8 mM EGTA; MEL, murine erythroleukemia.

native complement pathway, recognition by some I-type lectins, and on tissue homing in intact mice. We find that in each process, 9-*O*-acetylation has a substantial influence.

EXPERIMENTAL PROCEDURES

Materials—Most of materials used were obtained from Sigma .The following materials were obtained from the sources indicated. Alkaline phosphatase-conjugated goat anti-human IgG antibody was from Bio-Rad; fluorescein isothiocyanate-conjugated goat anti-human IgG and phycoerythrin-conjugated goat anti-human IgG were from CalTag Laboratories (South San Francisco, CA); fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG antibody and *Arthrobacter ureafaciens* neuraminidase were from Calbiochem; specific anti-factor H antibody (sheep antiserum) was from Accurate Chemicals (Netherlands); diisopropyl fluorophosphate was from Aldrich; dimethyl sulfoxide (Me_2SO) was from Fisher; [^{3}H]thymidine (specific activity 50 mCi/mmol) was from ICN Radiochemicals (Costa Mesa, CA); fetal calf serum was from Hyclone (Logan, UT). Protein assays were determined with the bicinchoninic acid protein assay reagent kit (Pierce) using BSA as a standard. The *O*-sialoglycoprotease enzyme was a kind gift from Dr. Alan Mellors, University of Guelph, Canada. All other chemicals were of reagent grade or better and were obtained from commercial sources.

Cell Lines—Murine erythroleukemia (MEL) cells were obtained from George Palade at University of California San Diego (14, 15) and cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum.

Chimeric Proteins—The soluble chimeric protein CHE-Fc, consisting of the extracellular domain of influenza C hemagglutinin esterase fused to the Fc portion of human IgG_1, was generated and characterized as described elsewhere (13). The modified form CHE-FcD was generated by treating CHE-Fc with 1 mM diisopropyl fluorophosphate to inactivate the esterase as described (13). CHE-Fc specifically releases 9-*O*-acetyl esters from sialic acids, whereas CHE-FcD specifically recognizes and binds to 9-*O*-acetylated sialic acids (13). The CD22Rg chimera was prepared exactly as described previously (16), by stable expression in wild-type Chinese hamster ovary cells. Chimeric sialoadhesin was purified from a Chinese hamster ovary lec2 cell line that was stably transfected with a plasmid encoding a chimeric fusion protein of the outer three Ig domains of murine sialoadhesin fused to the Fc portion of human IgG. The original construct (Sn-Rg) has been described elsewhere (17) and was kindly provided by Paul Crocker (ICRF, Oxford, UK) and Sorge Kelm (University of Kiel, Germany).

Preparation of Normal Human Serum—Blood was collected from volunteers and allowed to clot at room temperature for 1 h. Serum was separated from the clot by centrifugation at 2000 rpm for 20 min and stored in aliquots at −70 °C until use.

Extraction of Proteins from Cultured Cells—Washed cell pellets were resuspended into a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitors (1 mM EDTA, 50 μg/ml leupeptin, and 4 μg/ml pepstatin) and incubated for 10 min at room temperature with occasional mixing. After high speed ultracentrifugation, the supernatant was saved for analysis.

Removal of 9-O-Acetyl Esters from Cell Surface Sialic Acids—1×10^6 MEL cells were resuspended in 100 μl of PBS, containing 0.02% NaN_3 (control cells), or 100 ml of PBS, containing 0.02% NaN_3 with 10 μg of CHE-Fc. After incubation at 37 °C for 60 min, the cells were washed three times in ice-cold PBS and then stained for flow cytometry analysis as described below.

Removal of Sialic Acids from Cell Surface Oligosaccharides—1×10^6 MEL cells were resuspended in 100 μl of 50 mM NaAc (pH 6.0) with 10 milliunits of *A. ureafaciens* sialidase at 37 °C for 2 h, and the cells were washed three times in ice-cold PBS to remove the residual enzyme.

O-Sialoglycoprotease Treatments—Proteins (40-μg aliquots) from MEL cells were incubated with 2.5 milliunits of enzyme in 100 mM HEPES (pH 7.4), in the presence of BSA (stabilizer) for 2 h at 37 °C. For treatment of cell surface molecules, MEL cells (100 μl, 10^6 cells/ml) in Hanks' balanced saline solution, 1% fetal calf serum, 0.1% NaN_3, and 10 mM Neu2en5Ac (sialidase inhibitor) were incubated for 2 h at 37 °C in the presence or absence of 5 milliunits of *O*-sialoglycoprotease. The cells were then washed with Hanks' balanced saline solution, 1% fetal calf serum, 0.1% NaN_3 prior to the cell lysis assay.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Proteins were separated by SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), and the blots incubated overnight at 4 °C with sialoadhesinRg (10 μg/ml) in PBS, 1% BSA. Glycoproteins reacting with the chimera were colorimetrically detected with a goat anti-human IgG antibody conjugated with alkaline phosphatase and developed with *p*-nitrophenyl phosphate.

Growth and Differentiation of MEL Cells—Differentiation was induced by growing MEL cells (starting inoculum of 5×10^5 cells/ml) in the presence of 2% Me_2SO in complete medium for 3 days. The extent of spontaneous differentiation of MEL cells was determined using benzidine staining to detect hemoglobin accumulation.

Staining of MEL Cells for Flow Cytometry Analysis—MEL cells were washed three times with 1% BSA in PBS and incubated in the same buffer with a mixture of fluorescein isothiocyanate-conjugated goat anti-human IgG and CHE-FcD or phycoerythrin-conjugated goat anti-human IgG and sialoadhesinRg for 2 h at 4 °C. Control cells were incubated with similar amounts of human IgG_1 in 1% BSA in PBS. Cells were washed once in PBS, fixed in 2% formaldehyde in PBS, and analyzed by flow cytometry using a Becton Dickinson FACScan instrument. Intact cells were gated based on their forward and side scattering characteristics.

Cell Killing Assay—Activation of the human alternative pathway was measured by direct lysis of MEL cells in dilutions of normal human serum. The serum was diluted with Gelatin Veronal Buffer containing Mg^{2+} and EGTA (GVB-Mg-EGTA, Sigma) to prevent any contribution by the classical complement pathway (18–20). MEL cells (1×10^6 in 0.1 ml of GVB-MG-EGTA, buffer) were added to serially diluted serum. The reaction mixtures were incubated for 45 min at 37 °C with agitation. 1 ml of PBS was added to each sample, and the percentage lysis of the MEL cells was determined by trypan blue staining. The reagent blank showed no lysis; 100% lysis was obtained with the addition of 1 ml of distilled H_2O to 1×10^6 MEL cells.

Factor H Protein Binding Assay (21, 22)—MEL cells (1×10^6 in 100 μl of GVB^{2+} buffer) were incubated with normal human serum (1:1 in PBS) at 37 °C for 15 min. The cells were washed twice with PBS, 1% BSA and resuspended in 100 μl of sheep anti-human factor H antibody (30 μg/ml) in PBS, 1% BSA. Samples were incubated on ice for 1 h, washed with PBS, 1% BSA, and resuspended in 100 μl of fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG antibody (1:50). After further incubation on ice for 1 h, cells were washed twice, resuspended in 500 μl of PBS, 3.7% formaldehyde, and analyzed for bound factor H protein by measuring fluorescence intensity on a FACScan instrument. The effect of de-*O*-acetylation and de-sialylation on factor H protein binding to MEL cells was studied by prior treatment of the cells with esterase (CHE-Fc) or sialidase, respectively, as described above.

Tissue Homing Experiment—2×10^5 MEL cells were labeled with 100 μCi of [^{3}H]thymidine for 3 days and chased in normal media overnight. The labeled cells were washed with PBS, suspended in 100 μl of PBS, and treated with CHE-Fc/or sham-treated at 37 °C for 1 h. 100 μl of the treated and sham-treated MEL cells were injected into the tail vein of 8-week-old female DBA/2J mice (Jackson Laboratory, Bar Harbor, ME). After 2 h, mice were sacrificed under anesthesia, and different organs were collected for analysis. Femoral bone marrow, spleen, liver, lungs, brain, thymus, lymph nodes, heart, and kidneys were removed and homogenized in PBS containing 1% Triton X-100, using a Polytron (Kinematica, Switzerland). The extracts of different organs were analyzed for presence of ^{3}H radioactivity as an index of presence of MEL cells (0.5 ml of extract/20 ml of scintillation fluid). Under these conditions, counting efficiency was comparable among the different extracts.

RESULTS AND DISCUSSION

MEL Cells Are Sensitive to Lysis by the Alternative Pathway of Complement—As shown in Fig. 1*A*, MEL cells are sensitive to lysis by normal human serum. Such spontaneous lysis by heterologous serum can be mediated by either the classical (antibody-dependent) or the alternative (antibody-independent) pathways of complement activation. The specific involvement of the alternative pathway in this lysis is indicated by the use of GVB-Mg-EGTA buffer to prevent any contribution of the classical complement pathway (18–20) and by the striking effects of dilution (Fig. 1*A*). This is confirmed by the finding that human serum deficient in the alternative pathway factor B does not kill cells (Fig. 1*B*). Previous studies have shown that lysis of murine erythrocytes by human serum occurs via the alternative pathway (10) and that the extent of lysis roughly correlated with the level of 9-*O*-acetylation of sialic acids (10). Although not proven directly, it was suggested that the *O*-

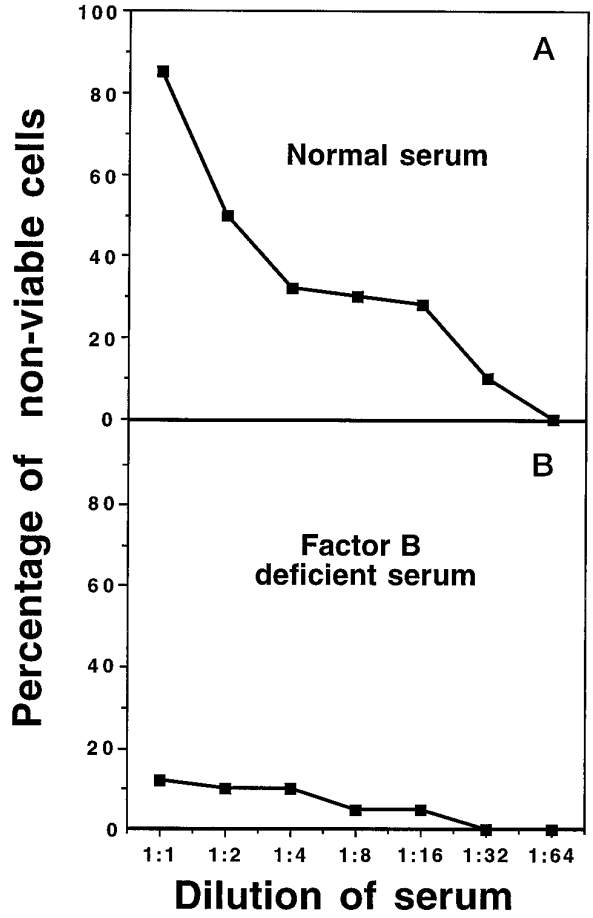

FIG. 1. **MEL cells are sensitive to lysis by the alternative pathway of complement.** The treatment of MEL cells with serum containing complement is described under "Experimental Procedures." The lysis of cells was determined by trypan blue staining. *A,* effects of serum dilution with GVB-Mg-EGTA buffer on the lysis of MEL cells. Under these conditions, the alternative pathway of complement is known to be active and to be sensitive to dilution. *B,* MEL cells cannot be lysed by factor B-deficient serum. Factor B is a critical component of the alternative pathway.

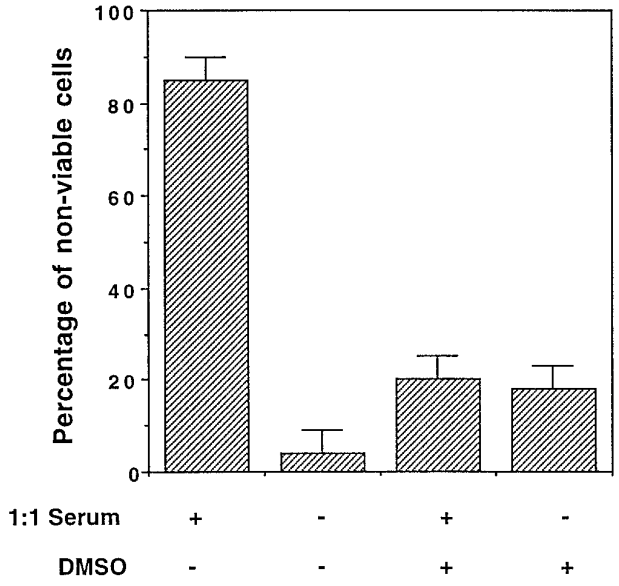

FIG. 2. **Differentiation of MEL cells gives protection from alternative pathway lysis.** MEL cells were grown in the presence or absence of 2% Me_2SO (*DMSO*) for 3 days and studied for lysis by 1:1 diluted serum as described under "Experimental Procedures." Following incubation for 45 min at 37 °C with agitation, 1 ml of PBS was added, and cell lysis was determined by trypan blue staining.

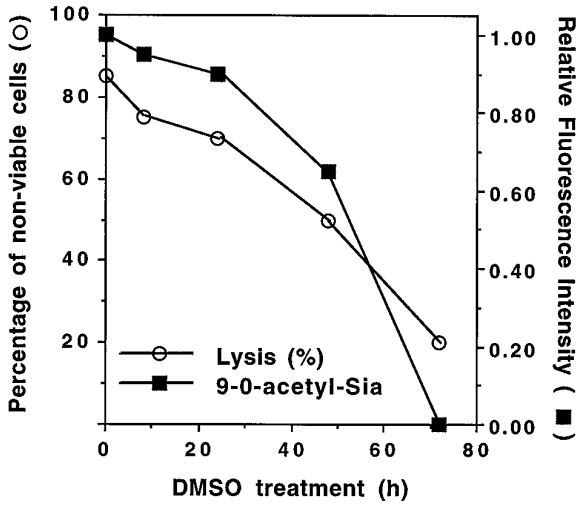

FIG. 3. **Protection from lysis upon differentiation correlates with loss of 9-*O*-acetylation.** MEL cells were grown in the presence of 2% Me_2SO (*DMSO*) for increasing periods and studied for lysis by 1:1 diluted serum as described under "Experimental Procedures." Aliquots of the same batches of MEL cells were stained with CHE-FcD and analyzed by flow cytometry to determine the expression of cell surface 9-*O*-acetylation. The latter data are presented as the relative mean fluorescence intensity of the signal seen.

acetyl groups were abrogating the normal function of sialic acid in restricting the alternative pathway of complement lysis. This type of restriction is caused by the regulatory factor H (18–20, 23), a soluble factor in serum which is believed to bind to surfaces via the intact exocyclic (C_7–C_8–C_9) side chain of sialic acids (18–20, 23). Since MEL cells also have cell surface 9-*O*-acetylation, we considered the possibility that a similar mechanism might be operating in this case.

Differentiation of MEL Cells Causes Resistance to Alternative Pathway Lysis Which Correlates with Loss of 9-O-Acetylation of Sialic Acids—In the preceding paper (12), we showed that induced differentiation of MEL cells is accompanied by the loss of cell surface 9-*O*-acetylation. As shown in Fig. 2, such differentiated MEL cells become resistant to human serum-mediated lysis, even when exposed to a high concentration (1:1 dilution) of serum. This could potentially be a direct result of the marked loss of cell surface 9-*O*-acetylation which should allow increased binding of factor H. However, differentiation is a complex process, and this loss of sensitivity could also be due to some unrelated molecular changes. To address this concern, we studied the time dependence of changes in sensitivity to lysis during the course of differentiation, in relationship to the density of cell surface 9-*O*-acetylation, as measured by flow cytometry with the FcD probe. As shown in Fig. 3, the extent of loss of cell surface 9-*O*-acetylation over time following the induction of differentiation correlates well with the increasing resistance to lysis.

Resistance to Alternative Pathway Lysis Can Also Be Obtained By Enzymatic Removal of 9-O-Acetyl Groups from Sialic Acids—To more directly address the role of cell 9-*O*-acetylation in complement sensitivity of MEL cells, we used two ways to remove cell surface 9-*O*-acetyl groups. In the preceding paper (12), we showed that treatment with the enzyme *O*-sialoglycoprotease selectively removes the cell surface mucins carrying *O*-acetylated sialic acids. As shown in Fig. 4*A*, this treatment also gave protection from lysis. An even more selective removal of 9-*O*-acetyl groups can be achieved by treatment with the recombinant soluble influenza CHE-Fc esterase. As shown in

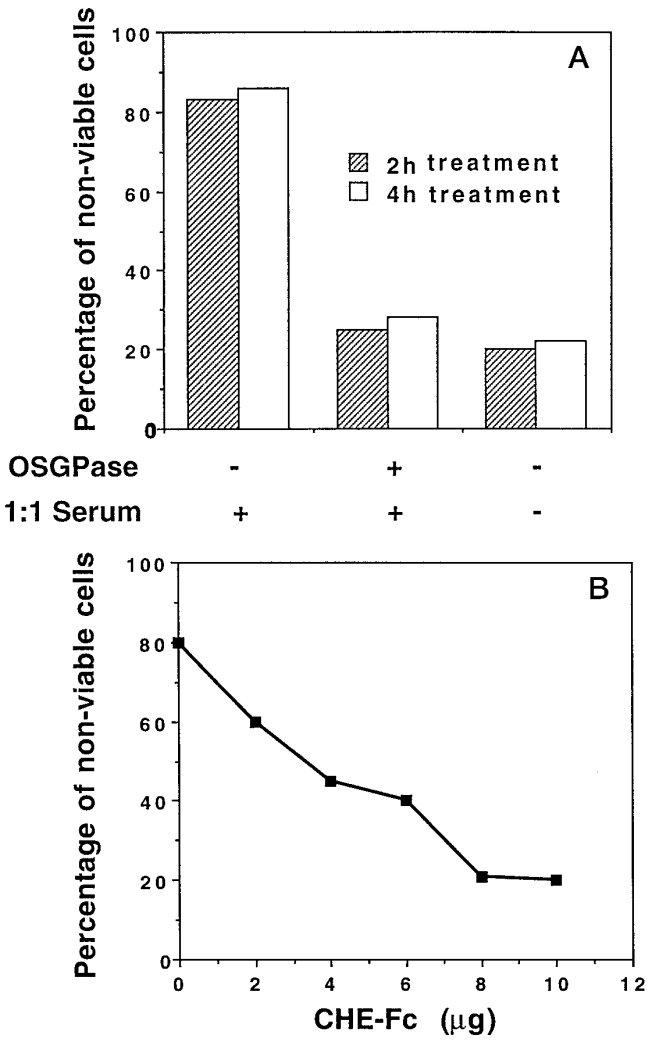

FIG. 4. **Removal of 9-*O*-acetyl groups from undifferentiated cells gives protection from complement lysis.** Undifferentiated MEL cells were incubated at 37 °C with various enzymes and studied for lysis by 1:1 diluted serum as described under "Experimental Procedures." *A, O*-sialoglycoprotease (*OSGPase*) (5 milliunits) for 2 or 4 h, respectively. *B,* incremental amounts of CHE-Fc for 1 h.

Fig. 4*B*, treatment with increasing concentrations of this enzyme also resulted in increasing resistance to lysis.

Increase in Cell Surface 9-O-Acetylation of Sialic Acids Caused by Nocodazole Correlates with Sensitivity to Alternative Pathway Lysis—In the accompanying paper (12), we also showed that treatment with nocodazole causes an accumulation of cell surface 9-*O*-acetylation on MEL cells, apparently by preventing endocytosis and turnover of the cell surface molecules. As shown in Fig. 5, this treatment is accompanied by the increased sensitivity to killing by diluted serum. Taken together with the effects of induced differentiation and enzymatic treatments described above, these data directly demonstrate for the first time that 9-*O*-acetylation can regulate the alternative pathway of complement. The prior work of others indicates that the exocyclic side chain of sialic acids is required for binding of factor H of the alternative pathway (18–20, 23), which normally restricts amplification of the C3bBb complex on surfaces (20, 23–25). Thus, it is reasonable to suggest that substitution of this side chain with a bulky *O*-acetyl ester abrogates this restrictive function, allowing amplification to proceed.

Factor H Direct Binding to MEL Cells Is Dependent on Sialic Acids and Blocked by O-Acetylation at Their Exocyclic Chain—To test the hypothesis mentioned above, we studied direct binding of human factor H to MEL cells, exploiting different conditions that allowed us to modify cell surface sialylation and acetylation of sialic acids. Factor H binding was studied by incubating cells for 15 min at 37 °C with human serum, which has ~0.5 mg/ml factor H (25). After this incubation, factor H binding was monitored using anti-factor H antibody and an adequate fluoresceinated conjugate by flow cytometry. As shown in Fig. 6, factor H binding increased significantly upon Me_2SO-induced differentiation. As we discussed in the accompanying paper (12), Me_2SO-induced differentiation was followed by increase in wheat germ agglutinin binding on the cell surface and decrease of sialic acid *O*-acetylation in sialomucins. Factor H binding to MEL cells is dependent on sialic acids, since it could be abrogated by sialidase treatment (Fig. 6, sialidase treatment also caused a loss of wheat germ agglutinin reactivity, indicating the efficiency of the treatment, data not shown). De-*O*-acetylation of MEL sialic acids was achieved by esterase treatment (CHE-Fc treatment). Factor H binding in esterase-treated cells was significantly increased, suggesting that the *O*-acetyl groups can mask factor H recognition or binding to sialic acid-rich surfaces as those represented by MEL cells. These results correlate well with the increased sensitivity of both Me_2SO-differentiated and esterase-treated MEL cells to the lytic activity of the alternative pathway of complement. They also illustrate the role of sialic acid modifications in modulating the binding activity of sialic acid binding proteins (in this case the complement factor H) and thus their function.

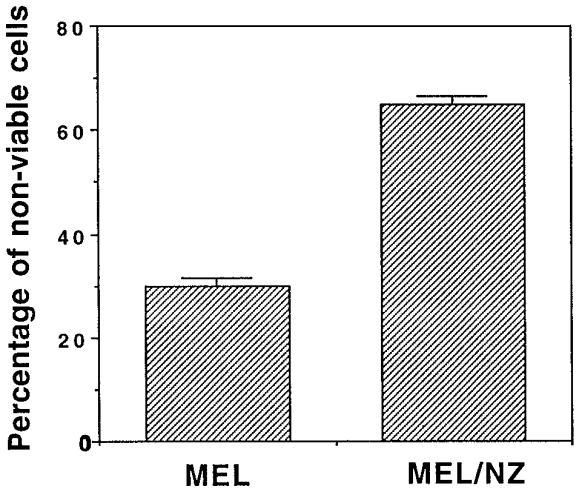

FIG. 5. **Nocodazole-induced enhancement of cell surface 9-*O*-acetylation results in increased susceptibility to complement lysis.** MEL cells were cultured in the presence of nocodazole (1 mg/ml) for 24 h. The nocodazole-treated or sham-treated MEL cells were added to 1:4 diluted serum and incubated at 37 °C for 1 h, and the lysis of cells was determined by trypan blue exclusion. Under these conditions an increase in cell surface 9-*O*-acetylation was detected with CHE-FcD staining analyzed by flow cytometry.

Binding of MEL Cells to the I-type Lectin Sialoadhesin Is Enhanced by Decrease of 9-O-Acetylation—Recently, several other sialic acid-binding molecules have been found in vertebrate systems. One group is a newly recognized family of mammalian lectins belonging to the immunoglobulin superfamily (I-type lectins), which include sialoadhesin and CD22 (8). Sialoadhesin is an adhesion molecule restricted to macrophages, and its highest expression is found in hematopoietic and lymphoid tissues like bone marrow, spleen, and lymph nodes. Sialoadhesin mediates cell adhesion by binding to cell surface glycoconjugates terminating in Siaα2–3Galβ1–3GalNAc and/or Siaα2–3Galβ1–3(4)GlcNAc and has been proposed to play a

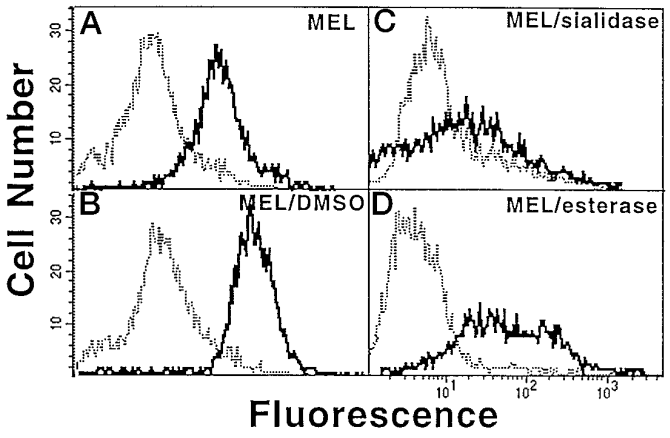

FIG. 6. **Sialic acids, but not 9-*O*-acetyl-sialic acids, support direct binding of complement factor H to MEL cells.** Control and treated MEL cells were incubated with normal human serum (containing ~0.5 mg/ml human factor H) for 15 min at 37 °C. Factor H binding to MEL was measured with anti-factor H antibodies by flow cytometry as described under "Experimental Procedures." The *solid line* indicates factor H binding, and the *dotted line* indicates background seen with secondary antibody alone. Me_2SO (*DMSO*) and esterase treatment, which decrease the amount of *O*-acetylated sialic acids on MEL cell surface, showed an increase in factor H binding. Conversely, treatment of MEL cells with sialidase abrogated factor H-specific binding almost completely.

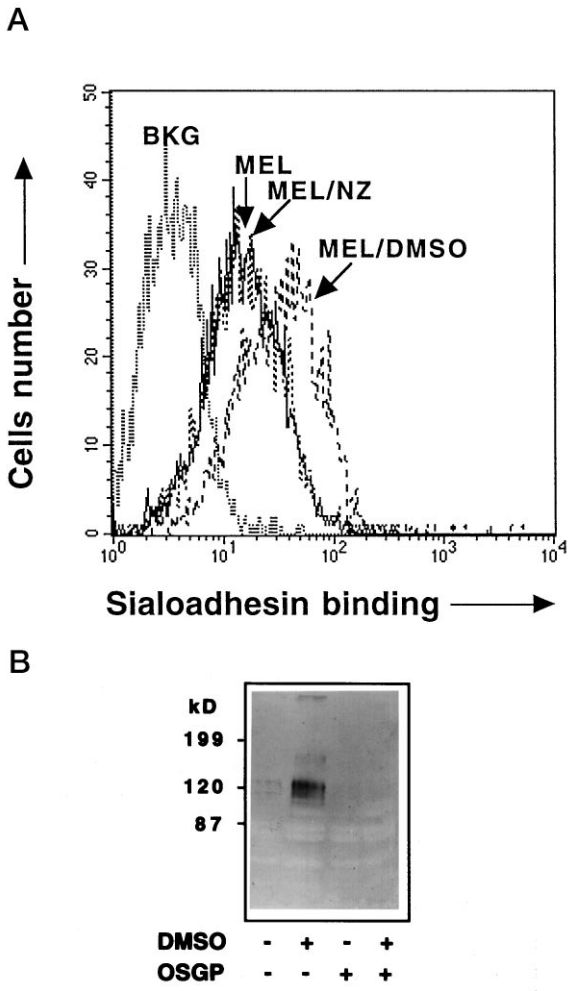

FIG. 7. **Me_2SO-induced differentiation of MEL cells increases binding of the I-type lectin sialoadhesin.** *A*, MEL cells cultured in complete medium with 2% Me_2SO (*DMSO*), with nocodazole (*NZ*) (1 mg/ml), or with no addition (control) at 37 °C for 72 h (24 h for nocodazole treatment). The cells were washed with PBS and stained with a recombinant soluble chimeric form of sialoadhesin followed by a phycoerythrin-conjugated secondary antibody and analyzed by flow cytometry as described under "Experimental Procedures." (*BKG,* staining with secondary antibody alone). *B*, MEL cells were cultured with or without 2% Me_2SO (*DMSO*) in complete medium. After 72 h, the cells were harvested, and total proteins were extracted as described under "Experimental Procedures." Aliquots of the extracts (40 μg of protein) were treated with 2.5 milliunits of *O*-sialoglycoprotease (*OSGP*) in 100 mM HEPES (pH 7.4), in the presence of BSA (stabilizer) for 2 h at 37 °C. Enzyme-treated or sham-treated protein (20 μg each) were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to blots, which were probed with a recombinant soluble chimeric form of sialoadhesin and an alkaline phosphate-conjugated secondary antibody. Binding was detected by developing with nitro blue tetrazolium and bromochloroindolyl phosphate substrate.

role in the interactions of macrophages with blood cells (5). Earlier studies have shown that sialoadhesin can bind to MEL cells (26). CD22 is known to bind selectively to the sequence Siaα2–6Galβ1–4GlcNAc. Since I-type lectins usually require the side chain of sialic acids for recognition (5–7, 17, 27, 28), we investigated the possibility that the cell surface 9-*O*-acetylation on these cells might be restricting binding of sialoadhesin. Indeed, as shown in Fig. 7*A*, the binding of recombinant soluble murine sialoadhesin to MEL cells was increased upon differentiation, shown earlier to be accompanied by loss of cell surface 9-*O*-acetylation. In keeping with this, sialoadhesin binding to total sialoglycoconjugates on a Western blot is markedly increased after differentiation. Interestingly, the increased binding appears to be confined to the *O*-sialoglycoprotease-sensitive sialomucins (Fig. 7*B*). Some binding of CD22Rg was also noted but no increase occurred upon CHE-Fc esterase treatment (data not shown). This is in contrast to some other cell types such as lymphocytes and hepatocytes, in which *O*-acetylation can mask CD22 ligands (4).

Removal of Cell Surface 9-O-Acetyl Groups Enhances Sialoadhesin Binding—Although differentiation of MEL cells gave a decrease of 9-*O*-acetylation and increase of sialoadhesin binding, the two phenomena could still be unrelated. To directly address this issue, we treated MEL cells with the CHE-Fc esterase and then studied sialoadhesin binding by flow cytometry. As shown in Fig. 8, this treatment resulted in a >1 log increase in the mean fluorescence intensity of sialoadhesin binding. Taken together, these data show that 9-*O*-acetylation abrogates recognition of sialomucins by sialoadhesin. They also fit with recent studies by others (7) in which exposure of murine erythrocytes to whole virions of influenza C increased interactions with macrophages carrying sialoadhesin.

Removal of Cell Surface 9-O-Acetylation Alters in Vivo Tissue Homing of MEL Cells—Upon injection into syngeneic DBA/2J mice, MEL cells are known to colonize organs like the liver, spleen, and bone marrow, eventually resulting in death from leukemic infiltration (11). However, the adhesive mechanisms involved in the initial homing of injected cells are unknown. Since sialoadhesin is known to be present in lymph nodes, spleen, bone marrow, and liver, we reasoned that specific interactions could occur with injected MEL cells, which in turn could be affected by the level of 9-*O*-acetylation. We therefore labeled MEL cells in culture with [^{3}H]thymidine, injected them into the tail veins of DBA/2 mice, and studied the short-term homing of the injected cells by harvesting organs after 2 h. As shown in Fig. 9, a major fraction of the injected cells was recovered in the spleen and liver, and this fraction was significantly increased by prior treatment of the cells with the CHE-Fc esterase. Some changes were also noted in recovery in other organs. However, the only other statistically significant change was the decrease in homing to the brain. This *in vivo* study involves complex variables and is obviously subject to many interpretations. Since induced differentiation of cells is

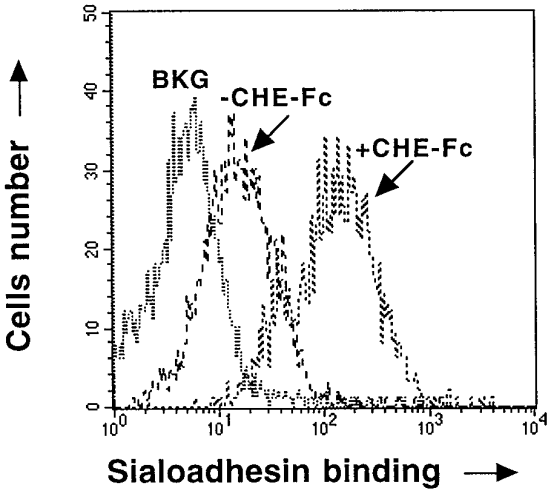

FIG. 8. **Removal of cell surface 9-*O*-acetyl groups results in increased binding of sialoadhesin.** Confluent MEL cells were washed and treated with CHE-Fc (5 μl of 1 mg/ml) for 1 h at 37 °C. The CHE-Fc-treated or sham-treated cells were washed with PBS and stained with a recombinant soluble chimeric form of sialoadhesin followed by a phycoerythrin-conjugated secondary antibody and analyzed by flow cytometry as described under "Experimental Procedures" (*BKG,* background; secondary antibody alone).

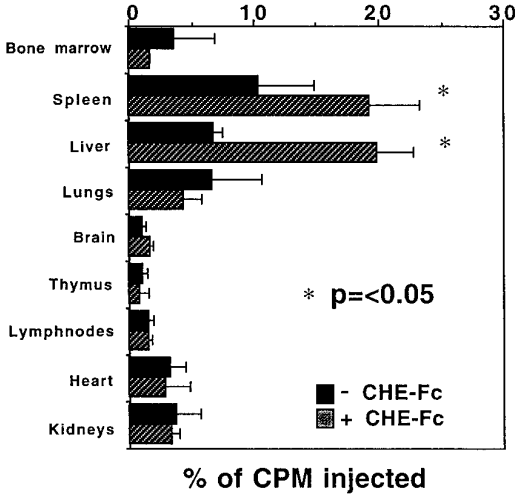

FIG. 9. **Removal of cell surface 9-*O*-acetyl groups increases *in vivo* homing to some mouse tissues.** MEL cells were labeled with [^{3}H]thymidine for 3 days under standard culture conditions allowing exponential growth, as described under "Experimental Procedures." Cells were then washed and treated with CHE-Fc (5 μl of 1 mg/ml) for 1 h at 37 °C. Aliquots (10^6) of treated or sham-treated cells were then injected into the tail vein of syngeneic DBA-2 mice. After 2 h, the mice were anesthetized and exsanguinated by cardiac puncture. Extracts of the different organs collected were analyzed for the presence of ^{3}H label as described under "Experimental Procedures." The distribution of MEL cells in different organs was expressed as a percent of the total radioactivity injected. Results represent means of data from three mice in each group ± S.D. Significant differences in distribution ($p < 0.05$ as determined using Student's *t* test for paired samples) are indicated by an *asterisk* (*).

also a complex process, we did not study the effects of this manipulation on homing. Regardless, the effects of the direct enzymatic esterase treatment indicate that the extent of cell surface 9-*O*-acetylation can modulate the tissue distribution of the erythroleukemia cells within the intact animal.

CONCLUSIONS AND PERSPECTIVES

Many potential functions have been attributed to the 9-*O*-acetylation of sialic acids (2, 3), but few have been directly proven. Here we have developed a system wherein some of these hypotheses can be tested. Thus, we have shown that 9-*O*-acetylation can abrogate the normal function of the exocyclic side chain of Sia in preventing activation of the alternative complement pathway (18–20, 23). Addition of a bulky acetyl group caused a loss of binding of factor H, a protein which normally restricts the amplification loop of alternative pathway activation by dissociation of Bb from C3b and by acting as a cofactor for factor I proteolysis of C3b (18–20, 23–25). We have also shown here an example of how an *O*-acetyl group can block recognition of sialic acid by an I-type lectin, sialoadhesin. In other work, it has been shown that influenza A and B virus hemagglutinin recognition of sialic acids cannot tolerate *O*-acetyl substitution of the side chain (29). In each case, *O*-acetylation can thus potentially act as a physiological modulator of binding. On the other hand, some viral and animal hemagglutinins require a 9-*O*-acetyl group for recognition.

There are many other intriguing observations that suggest that 9-*O*-acetylation may modulate biological phenomena involving sialic acids. For example, indirect observations suggest the *O*-acetylation of sialic acids on murine erythrocytes may confer resistance to malarial parasite binding (30). Also, in adult mice subjected to repeated bleeding, the level of 9-*O*-acetylation in circulating red blood cells appeared to increase (10). Some thymocytes and mature lymphocytes have *O*-acetylated Sias, whereas others do not (4, 31–33). 9-*O*-Acetylation is also selectively expressed at high levels on certain lymphocyte populations (32–36), and T-cells of patients with various malignancies are said to acquire high levels of *O*-acetylation (37). *O*-Acetylated Sias are often found as developmentally regulated components of neuroectodermal gangliosides (38–41), suggesting a role in morphogenesis. The approaches used in the present study might be adapted to successfully elucidate the significance of some of these phenomena as well.

Acknowledgments—We thank Steffen Thiel and Sandra Diaz for helpful discussions and careful review of the manuscript.

REFERENCES

1. Schauer, R. (ed) (1982) *Sialic Acids: Chemistry, Metabolism and Function, Cell Biology Monographs,* Vol. 10, Springer-Verlag Inc., New York
2. Schauer, R. (1991) *Glycobiology* **1,** 449–452
3. Varki, A. (1992) *Glycobiology* **2,** 25–40
4. Sjoberg, E. R., Powell, L. D., Klein, A., and Varki, A. (1994) *J. Cell Biol.* **126,** 549–562
5. Crocker, P. R., Mucklow, S., Bouckson, V., McWilliam, A., Willis, A. C., Gordon, S., Milon, G., Kelm, S., and Bradfield, P. (1994) *EMBO J.* **13,** 4490–4503
6. Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., De Bellard, M. -E., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P., and Crocker, P. R. (1994) *Curr. Biol.* **4,** 965–972
7. Kelm, S., Schauer, R., Manuguerra, J.-C., Gross, H.-J., and Crocker, P. R. (1994) *Glycoconjugate J.* **11,** 576–585
8. Powell, L. D., and Varki, A. (1995) *J. Biol. Chem.* **270,** 14243–14246
9. Varki, A., Hooshmand, F., Diaz, S., Varki, N. M., and Hedrick, S. M. (1991) *Cell* **65,** 65–74
10. Varki, A., and Kornfeld, S. (1980) *J. Exp. Med.* **152,** 532–544
11. Marks, P. A., and Rifkind, R. A. (1978) *Annu. Rev. Biochem.* **47,** 419–448
12. Shi, W.-X., Chammas, R., and Varki, A. (1996) *J. Biol. Chem.* **271,** accompanying manuscript, 408631 31517–31525
13. Klein, A., Krishna, M., Varki, N. M., and Varki, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7782–7786
14. Dolci, E. D., and Palade, G. E. (1989) *J. Cell Sci.* **93,** 191–197
15. Ulmer, J. B., and Palade, G. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6992–6996
16. Hanasaki, K., Varki, A., and Powell, L. D. (1995) *J. Biol. Chem.* **270,** 7533–7542
17. Nath, D., van der Merwe, P. A., Kelm, S., Bradfield, P., and Crocker, P. R. (1995) *J. Biol. Chem.* **270,** 26184–26191
18. Kazatchkine, M. D., Fearon, D. T., and Austen, K. F. (1979) *J. Immunol.* **122,** 75–81
19. Nydegger, U. E., Fearon, D. T., and Austen, K. F. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 6078–6082
20. Meri, S., and Pangburn, M. K. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3982–3986
21. Ollert, M. W., David, K., Bredehorst, R., and Vogel, C. W. (1995) *J. Immunol.* **155,** 4955–4962
22. Avery, V. M., and Gordon, D. L. (1993) *J. Immunol.* **151,** 5545–5553
23. Michalek, M. T., Mold, C., and Bremer, E. G. (1988) *J. Immunol.* **140,** 1588–1594

24. Zipfel, P. F., and Skerka, C. (1994) *Immunol. Today* **15,** 121–126
25. Law, S. K. A., and Reid, K. B. M. (1995) *Complement*, IRL Press at Oxford University Press, New York
26. Fraser, I. P., and Gordon, S. (1994) *Eur. J. Cell Biol.* **64,** 217–221
27. Crocker, P. R., Freeman, S., Gordon, S., and Kelm, S. (1995) *J. Clin. Invest.* **95,** 635–643
28. Freeman, S. D., Kelm, S., Barber, E. K., and Crocker, P. R. (1995) *Blood* **85,** 2005–2012
29. Higa, H. H., Rogers, G. N., and Paulson, J. C. (1985) *Virology* **144,** 279–282
30. Klotz, F. W., Orlandi, P. A., Reuter, G., Cohen, S. J., Haynes, J. D., Schauer, R., Howard, R. J., Palese, P., and Miller, L. H. (1992) *Mol. Biochem. Parasitol.* **51,** 49–54
31. Schwarting, G. A., and Gajewski, A. (1983) *J. Biol. Chem.* **258,** 5893–5898
32. Kniep, B., Flegel, W. A., Northoff, H., and Rieber, E. P. (1993) *Blood* **82,** 1776–1786
33. Zimmer, G., Suguri, T., Reuter, G., Yu, R. K., Schauer, R., and Herrler, G. (1994) *Glycobiology* **4,** 343–350
34. Kniep, B., Claus, C., Peter-Katalinic, J., Monner, D. A., Dippold, W., and Nimtz, M. (1995) *J. Biol. Chem.* **270,** 30173–30180
35. Kniep, B., Peter-Katalinic, J., Flegel, W., Northoff, H., and Rieber, E. P. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1343–1349
36. Kamerling, J. P., Makovitzky, J., Schauer, R., Vliegenthart, J. F. G., and Wember, M. (1982) *Biochim. Biophys. Acta* **714,** 351–355
37. Stickl, H., Huber, W., Faillard, H., Becker, A., Holzhauser, R., and Graeff, H. (1991) *Klin. Wochenschr.* **69,** 5–9
38. Levine, J., Beasley, L., and Stallcup, W. (1984) *J. Neurosci.* **4,** 820–831
39. Constantine-Paton, M., Blum, A. S., Mendez-Otero, R., and Barnstable, C. J. (1986) *Nature* **324,** 459–462
40. Blum, A. S., and Barnstable, C. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8716–8720
41. Drazba, J., Pierce, M., and Lemmon, V. (1991) *Dev. Biol.* **145,** 154–163