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Research Paper

Modulating cell surface immunoreactivity by metabolic induction of unnatural carbohydrate antigens

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Abstract

Background: Sialic acid is a component of many tumor-associated oligosaccharide antigens. The repertoire of sialic acids presented by cells can be expanded to include unnatural variants by intercepting the sialic acid biosynthetic pathway with unnatural precursors. We explored whether unnatural cell surface sialosides produced by metabolism can act as neo-antigens and modulate the immunogenicity of cells.

Results: Immunization of rabbits with synthetic conjugates of an unnatural sialic acid bound to keyhole limpet hemocyanin produced significant titers of antibodies that were specific for the structurally altered sialic acid. The antibodies recognized cells that were fed the unnatural biosynthetic precursor, and were capable of directing complement-mediated lysis.

Conclusions: Structural alteration of sialic acids replaces a tolerized self-antigen with an antigenic determinant. Incorporation of unnatural sialosides into cell surface glycoconjugates through biosynthetic means can alter the immunoreactivity of cells, providing new possibilities for tumor immunotherapy.

Keywords: Sialic acid; Antibody; Immune response; Oligosaccharide; Tumor

1. Introduction

Cancer cells have long been known to express glycosylation patterns that are different from those found on the parent cells [1–4]. The structural characterization of these altered glycoforms has identified carbohydrate motifs associated with tumor tissue. Some are truly tumor-specific antigens that have not been found in normal tissue; other antigens are present in normal tissue, but over-expressed on tumor cells, and still others are displayed during fetal development, disappear in the adult, only to arise again during malignant transformation [3,5]. Sialic acids are components of many tumor-associated carbohydrate antigens [6–9]. As occupants of the terminal positions in numerous cell surface oligosaccharide structures, sialic acids are poised to interact with the immune system, and yet sialylation of cell surface oligosaccharides can mask underlying antigenic determinants [10,11]. This suggests at least one role for the hypersialylation of cell surface oligosaccharides observed in many forms of malignancy [12,13]. An unusual feature of sialic acids is their potential for species-specific structural elaboration by *O*-acetylation, *O*-methylation, *N*-deacetylation, hydroxylation, phosphorylation, or sulfation [11]. As a consequence, sialylated glycoproteins from one species can be antigenic when inoculated into another species, with at least part of the immune response being directed against the foreign sialoside [14,15]. Thus, while an organism's immune system has been induced to recognize its own sialylated glycoforms as self, subtle perturbations of the sialic acid structure can render the sialoforms antigenic.

We and others have shown that the natural diversity of sialic acids presented by cells can be expanded by intercepting the sialic acid biosynthetic pathway with structurally augmented non-natural biosynthetic substrates [16–20]. For example, cells can metabolize *N*-levulinoylmannosamine (ManLev) or the more bioavailable acetylated ManLev (**1**, Scheme 1) and express *N*-levulinoylneuraminic acid (SiaLev, **2**) on the cell surface [21,22]. The

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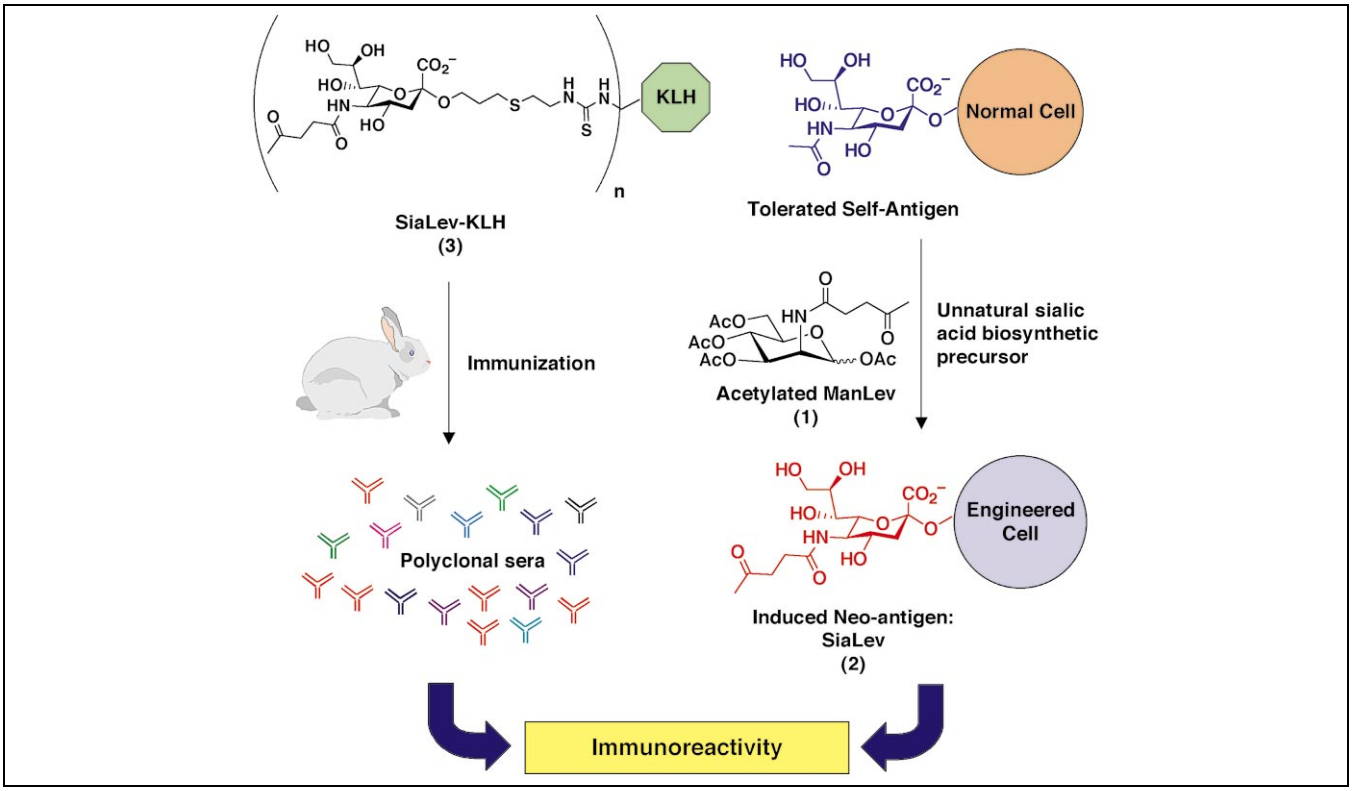

Scheme 1. A strategy for inducing immune recognition of cell surfaces based on unnatural sialic acid biosynthesis. Rabbits are immunized with unnatural sialic acids conjugated to keyhole limpet hemocyanin (**3**). Cells are induced to express SiaLev (**2**) as a component of their cell surface glycoconjugates by treatment with the metabolic precursor acetylated ManLev (**1**). Sera from the immunized rabbits are highly reactive only with SiaLev-expressing cells, while cells expressing natural sialosides are not reactive with the sera.

straightforward fashion in which structurally altered sialosides can be presented on cells prompted us to consider this as a possible mechanism to control their immunogenicity.

Cancer cells, while antigenic, are rarely immunogenic; they employ numerous mechanisms to evade immune detection during growth, engendering tolerance by the immune system [23,24]. Many attempts at generating anticancer vaccines have focused on breaking the immune tolerance for tumor-associated carbohydrate antigens [25–27]. An important theme that has arisen from these studies is the necessity of formulating the vaccine such that the antigen is presented to the immune system outside the context of the tumor. Synthetic antigens composed of multiple copies of a tumor-associated antigen bound to a nonself carrier protein have been shown to generate a high-titer antibody response against the carbohydrate antigen presented on the malignant cells [27,28]. In addition to its function as a scaffold for oligosaccharide presentation, the carrier protein also provides a T-cell stimulatory epitope which is necessary for the development of a complete immune response [29,30]. Chemical modification of carbohydrate structures has also been found to augment the antigenicity of oligosaccharide-based vaccines [27,31–34]. For example, modification of ganglioside-associated sialic acid residues by esterification, amidation, or reduction increases an antibody response [33,34]. However, the immune responses were rather specific, where most of the antibodies bound only the unnaturally modified structure with little cross-reactivity to native structures presented on tumor cells. While these results were not initially encouraging from the practical standpoint of developing effective anti-cancer vaccines, they suggest that introducing unnatural modifications into carbohydrate structures on immunogenic carrier proteins can potentiate the immune response against carbohydrate antigens.

Here we demonstrate that a synthetic neoglycoprotein comprising multiple copies of SiaLev conjugated to keyhole limpet hemocyanin (KLH, **3**), an immunogenic carrier protein, is highly antigenic in rabbits (Scheme 1). Specific, sustained, high-titer antibody responses directed against the SiaLev epitopes were induced following immunization with the neoglycoprotein. Cells treated with nanomolar to micromolar concentrations of acetylated ManLev were rendered highly reactive with the sera derived from these rabbits. The SiaLev induced on cells treated with acetylated ManLev was stable to aldehyde fixation, allowing characterization of the intracellular distribution of these unnatural glycoforms. Finally, we discovered that the immunogenicity of cells can be controlled with acetylated ManLev, as cells expressing the neo-antigen SiaLev were selectively lysed upon treatment with the anti-SiaLev

serum and complement. The ability to modulate the cell surface expression of SiaLev, and recruit immune system components to cells expressing this neo-antigen, suggests a new approach to immunotherapy based on unnatural sialic acid biosynthesis.

2. Results and discussion

2.1. Synthesis of antigens

The SiaLev–KLH antigen (**3**, Scheme 1) was synthesized as shown in Fig. 1. The allyl glycoside **5** was synthesized in three steps as previously described [35] from *N*-acetylneuraminic acid (**4**). In order to introduce the desired levulinoyl group as the *N*-acyl substituent, the *N*-acetamido group in compound **5** was converted to the acyl carbamate **6**. Treatment of compound **6** with catalytic sodium methoxide in methanol removed all acetyl groups, affording carbamate **7**. The amino group at C5 in **7** was liberated by treatment with trifluoroacetic acid (TFA), and immediately reacted with a mixed carbonic anhydride of levulinic acid (**8**) to yield compound **9**. The methyl ester was then saponified and the glycosidic *O*-allyl group was photochemically reacted with thioethanolamine to afford amine **10**. This unnatural sialic acid hapten was conjugated to the KLH by conversion of the free amino group to the isothiocyanate, followed by reaction with a concentrated solution of KLH to provide conjugate **3**. Approximately 60–70% of available lysines were modified with the epitope, resulting in roughly 160–190 SiaLev residues per molecule of KLH. Using the same procedure SiaLev was also conjugated to bovine serum albumin (BSA) for use in ELISAs.

2.2. Immunizations

Two New Zealand White rabbits were immunized and given boosts at regular intervals with the SiaLev–KLH conjugate. After each boost, whole serum was analyzed by ELISA using SiaLev–BSA as a substrate (Fig. 2a). The sera contained a significant titer of antibodies specific for the unnatural sialic acid. Importantly, no cross-reactivity was observed with native sialic acid in the form of a Sia$\alpha(2\rightarrow3)$Gal$\beta(1\rightarrow4)$GlcNAc–BSA (SiaLacNAc–BSA) conjugate. The titer of SiaLev-specific antibodies evoked following each boost of antigen was similar in both animals following repeated immunization (Fig. 2b). In addition, subtyping analysis revealed that a majority of the antigen-specific antibodies were of the IgG rather than the IgM subclass (data not shown), indicative of the recruitment of T-cell help in the induction of the immune response [27,36]. Thus, the SiaLev–KLH antigen used in this study appears to recruit the T-cell help required for a specific, potent immune response against an epitope (SiaLev) structurally similar to an abundant and ubiquitous self-antigen (sialic acid).

2.3. Antigenicity of cell surfaces

As demonstrated above, immunizations with SiaLev–KLH produced antibodies that bind immobilized SiaLev on a microtiter plate. We next evaluated whether the antibodies recognized SiaLev expressed within cell surface gly-

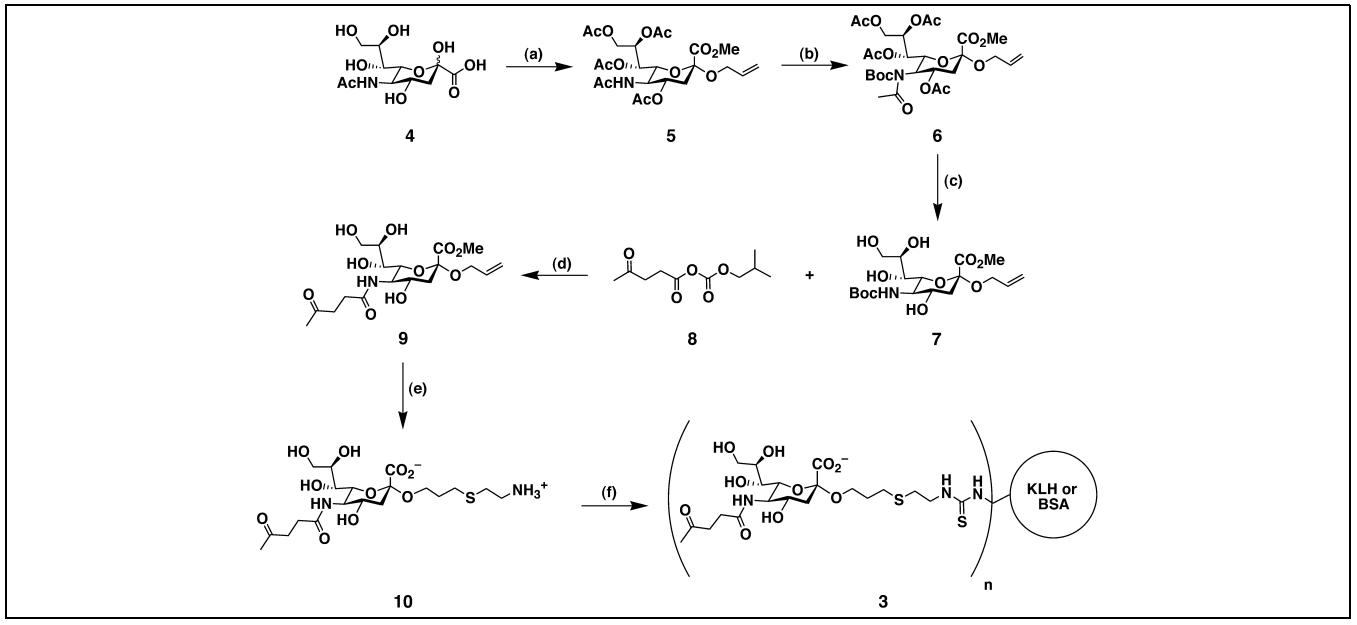

Fig. 1. Synthesis of SiaLev–KLH (**3**). (a) (i) MeOH, TFA; (ii) acetyl chloride, HCl; (iii) $AgClO_4$, allyl alcohol, 4 Å molecular sieves: 56% over three steps. (b) Di-*tert*-butyl dicarbonate, DMAP, TEA, THF: 57%. (c) NaOMe/MeOH: 93%. (d) (i) 10% TFA in CH_2Cl_2; (ii) NaOMe, **8**, methanol: 40% over two steps. (e) (i) NaOH; (ii) 2-aminoethanethiol hydrochloride, 254 nm light: 60% over two steps. (f) (i) thiophosgene, Na_2CO_3, 1:1 $H_2O/CHCl_3$; (ii) carrier protein (KLH or BSA), 300 mM $NaHCO_3$, pH 9: 60–70% of available lysines were conjugated.

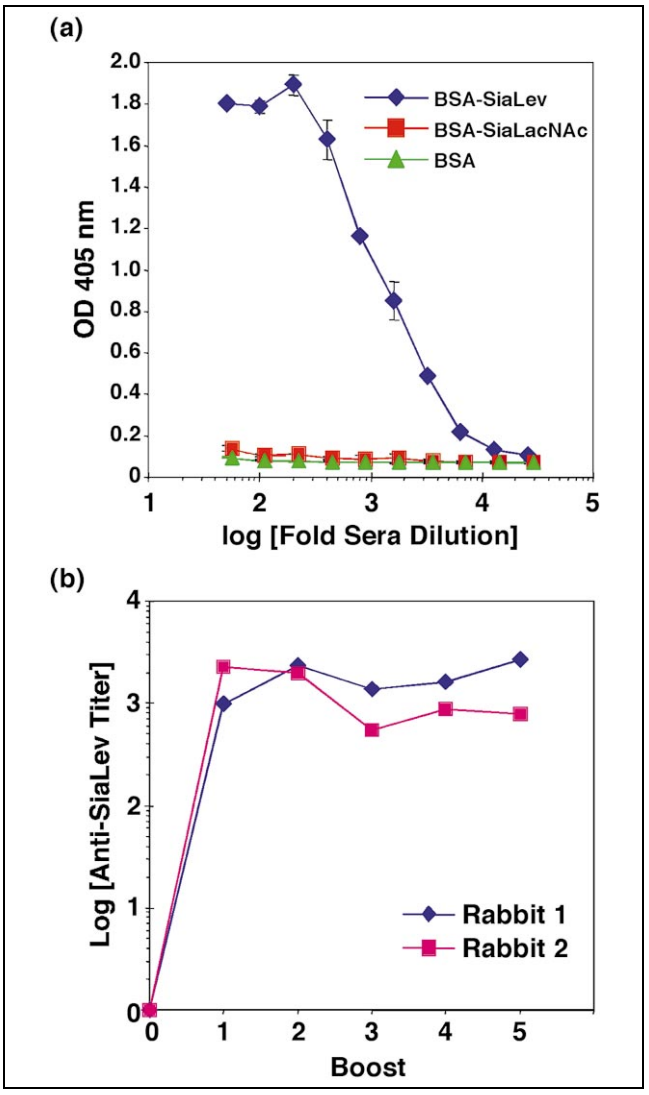

Fig. 2. Reactivity of rabbit antisera with SiaLev as determined by ELISA. (a) Reactivity of whole sera with SiaLev and SiaLacNAc. (b) Titer of sera from different rabbits following each antigen boost. Titer is defined as the dilution of serum which yields an optical density of half saturation in a set of serial dilutions such as that shown in (a).

coconjugates. Native sialic acid is found as a terminal sugar on both *N*- and *O*-linked glycoproteins, as well as glycolipids [37]. For these reasons, we chose to examine the reactivity of cell surfaces from cell lines known to present sialic acids in a variety of glycosylation environments. The three cell lines examined in this study were HeLa cells, which display both *N*- and *O*-linked sialoglycoproteins, Jurkat cells, which express sialic acid mainly in *N*-linked glycoproteins, and HL-60 cells, which display an abundance of *O*-linked sialoglycoproteins [21,38,39].

HeLa cells were cultured for 3 days in medium supplemented with 30 μM acetylated ManLev, washed and then labeled with diluted sera raised against SiaLev–KLH, and finally stained with a FITC-labeled secondary (anti-rabbit Ig) antibody. The degree of cell surface staining was assessed by flow cytometry (Fig. 3a). HeLa cells cultured in the presence of acetylated ManLev exhibited no detectable reactivity with pre-immunization sera from the animals. Likewise, HeLa cells treated with dilutions of anti-SiaLev sera (1:200) exhibited only background fluorescence staining when grown in medium without acetylated ManLev. However, cells that had been cultured in the presence of acetylated ManLev exhibited a marked increase in the immunoreactivity with anti-SiaLev sera. Similar results were observed with Jurkat and HL-60 cells (data not shown). In addition, the cell surface immunoreactivity of antisera pro-

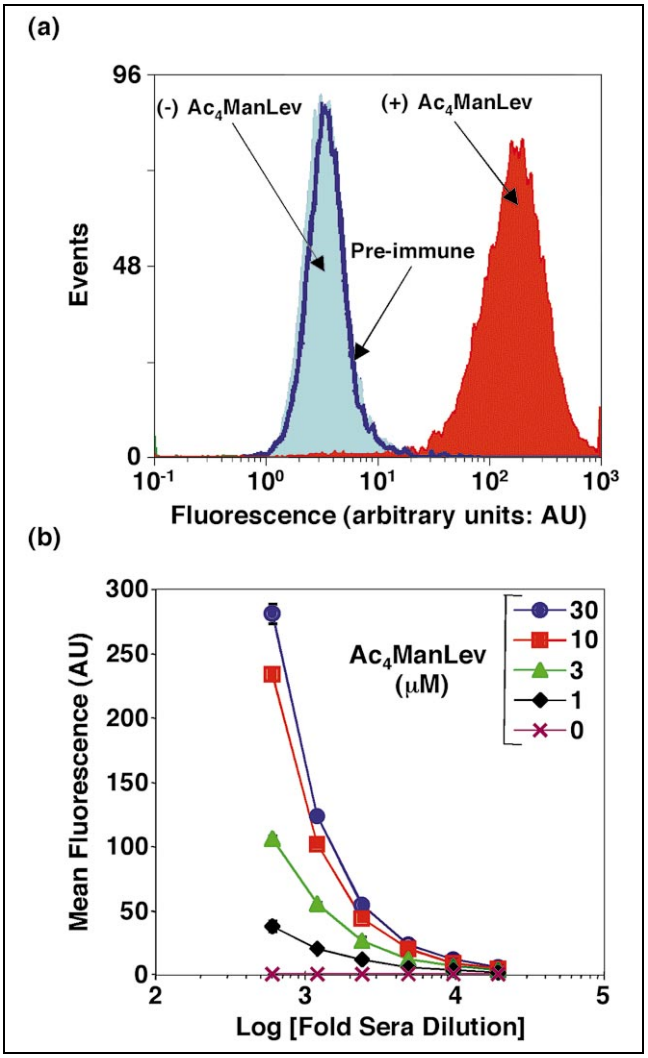

Fig. 3. Surface reactivity of live cells with SiaLev antisera assessed by flow cytometry. (a) Characteristic cell surface reactivity of cells determined by flow cytometry. Red: HeLa cells cultured with 30 μM acetylated ManLev (Ac_4ManLev) for 3 days, reacted with SiaLev antiserum, and RG-16-FITC (monoclonal mouse anti-rabbit Ig-FITC). Dark blue: HeLa cells cultured with 30 μM acetylated ManLev for 3 days, reacted with pre-immune serum from the same rabbit that produced the SiaLev antiserum, and RG-16-FITC. Light blue: HeLa cells cultured without ManLev, reacted with whole SiaLev antisera, and RG-16-FITC. (b) Cell surface reactivity of Jurkat cells cultured with different doses of acetylated ManLev (0–30 μM) and reacted with serial dilutions of sera (1:600–1:19 200). Reported are the mean fluorescence intensities, expressed in arbitrary units (AU), of populations of 10^4 cells. Error bars represent the high and low values of duplicate experiments.

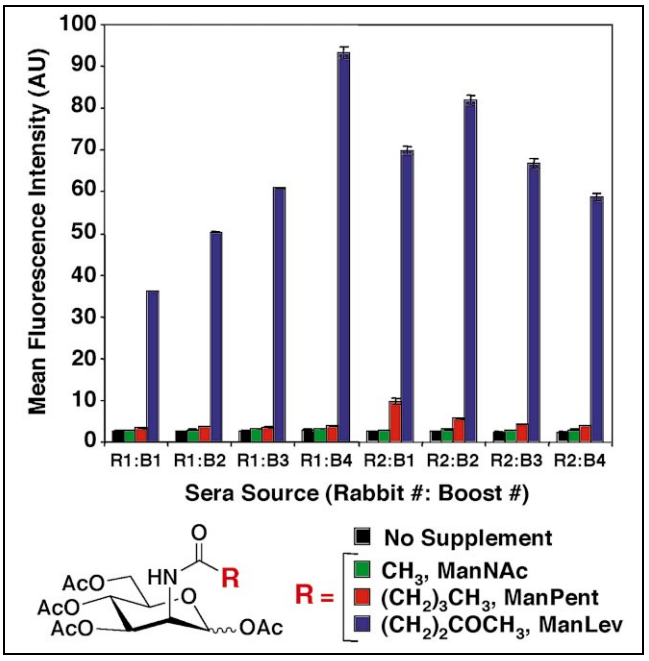

Fig. 4. SiaLev antiserum reactivity with cells treated with different sialic acid biosynthetic precursors. Mean fluorescence intensities were determined by flow cytometry analysis of HeLa cells cultured for 3 days with 30 μM acetylated ManNAc (green), ManPent (red), ManLev (blue), or no biosynthetic precursor (black). The cells were then washed and stained with sera from different immunized rabbits (R) collected after each antigen boost (B), followed by treatment with RG-16-FITC. Error bars represent the high and low values of duplicate experiments.

duced from successive boosts mirrored the titers observed by ELISA. The reactivity of the antisera with HeLa, HL-60, and Jurkat cell surfaces appeared to be insensitive to the different environments of their various sialosides. These results imply that the reactive antibodies in the sera are specific for the SiaLev epitope and are ignorant of both the linkage and type of glycoconjugate in which it is expressed.

The extent of antibody binding to SiaLev-expressing Jurkat cells was found to depend on both the amount of antibody added to a cell population and the concentration of acetylated ManLev present in the growth medium (Fig. 3b). Significant labeling of the cells was observed even when concentrations of acetylated ManLev were quite low (1 μM). Cells treated with higher concentrations of acetylated ManLev exhibited an extremely high amount of immunoreactivity, such that the antigen available for immunoreaction on the cell surface was apparently in excess of the antibody titer present in solution.

In order to determine the selectivity of the antiserum for SiaLev over other modified sialic acids, we treated HeLa cells with acetylated *N*-pentanoylmannosamine (ManPent), which differs from acetylated ManLev only by replacement of the carbonyl with a methylene group, and is known to be metabolized to cell surface sialosides [40]. Whole serum from four successive bleeds from both animals was analyzed for its capacity to bind cells treated with 30 μM acetylated ManNAc, ManPent, or ManLev (Fig. 4). The sera of rabbit 1 appeared to be entirely specific for SiaLev, as the SiaPent-expressing cells exhibited only background reactivity. However, the sera from rabbit 2 did exhibit minimal cross-reactivity with SiaPent-expressing cells which appeared to decrease with progressive antigen boosts, perhaps the result of affinity maturation. These results show that the sialic acid binding antibodies in the whole serum are specific for SiaLev.

2.4. Cellular distribution of SiaLev

In addition to the surface expression of SiaLev assessed by flow cytometry, we investigated the intracellular expression of SiaLev residues using affinity-purified SiaLev antisera. Serum that had been pre-cleared by passage through a sialic acid–agarose resin was applied to a SiaLev–agarose column. The immunoglobulins that bound the SiaLev–agarose resin were eluted, and used in the detection of intracellular SiaLev residues by fluorescence microscopy. HeLa cells cultured on glass coverslips were fixed, permeabilized, and stained with the affinity-purified sera (or a control antibody) followed by a fluorophore-labeled secondary antibody. The coverslips were then mounted on slides in a mounting medium containing DAPI, a DNA-specific stain [41] used to image the nuclei. Slides were

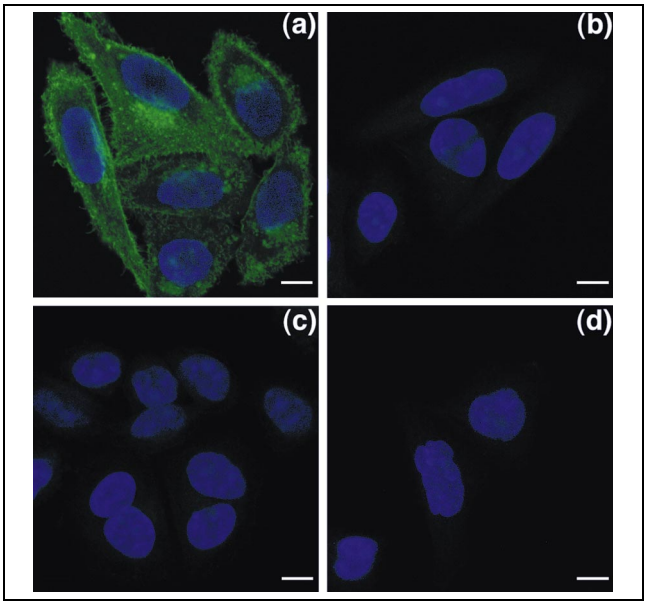

Fig. 5. Intracellular localization of SiaLev in fixed and permeabilized HeLa cells. (a) Cells cultured with 30 μM acetylated ManLev, stained with affinity-purified SiaLev antiserum, goat anti-rabbit IgG (H+L)-Alexa® Fluor™-488, and DAPI. (b) Cells cultured without acetylated ManLev, otherwise treated as in panel a. (c) Cells cultured with 30 μM acetylated ManNAc but without acetylated ManLev, otherwise treated as in panel a. (d) Cells cultured with 30 μM acetylated ManLev, treated with non-specific rabbit IgG, goat anti-rabbit IgG (H+L)-Alexa® Fluor™-488, and DAPI. Images shown are single 2 μm thick optical sections at 1000× magnification in a plane through the center of the nuclei. Scale bars represent 10 μm.

imaged by two-color laser scanning confocal microscopy (Fig. 5). An extensive continuum of SiaLev-containing vesicles leading from the Golgi (proximal to the nucleus) to the plasma membrane was revealed using the SiaLev-specific sera (Fig. 5a). By contrast, control cells treated with buffer (Fig. 5b), with acetylated ManNAc (Fig. 5c), or with non-specific rabbit IgG (Fig. 5d) showed no vesicular or plasma membrane staining. Thus, the processing of unnatural sialic acids through the secretory pathway can be tracked using specific antisera.

2.5. *Immunogenicity of cell surface SiaLev*

Finally, we investigated the ability to transform cell surfaces via unnatural sialic acid metabolism as a means of targeting cells for immune destruction. A major effector function of antibody binding to cell surfaces is the activation of complement against foreign particles or cells [42]. We investigated the activity of the antisera in stimulating antibody-dependent, complement-mediated cytolysis of cells that were induced to express SiaLev. Jurkat cells were cultured in media supplemented with 20 μM acetylated ManLev, 20 μM acetylated ManNAc, or no supplement. The cells were harvested and treated with affinity-purified anti-SiaLev serum or non-specific rabbit IgG as a control. Cells were then treated with rabbit serum complement or heat-inactivated complement as a control. Over 98% of cells that were treated with acetylated ManLev, anti-SiaLev serum, and complement were lysed in this assay (Fig. 6a). Removal of the antigen-specific sera or replacement with non-specific IgG induced only background lysis of these cells (15–20%). The background lysis was due to non-specific activity of complement, as heat-inactivating the complement prior to cell treatment nullified this effect. Lysis of the Jurkat cells was also dependent on their prior exposure to acetylated ManLev since the cell populations that were untreated or exposed to acetylated ManNAc exhibited only background levels of cytolysis (15–20%). These results demonstrate that SiaLev antiserum is a potent mediator of complement-dependent cytolysis and is selective for cells expressing the neo-antigen SiaLev.

The extent of complement-mediated lysis of Jurkat cells was found to depend on the dose of acetylated ManLev the cells were exposed to (Fig. 6b), presumably due to the resultant differences in cell surface antigen density. Maximal lysis was achieved when cells were cultured with concentrations of acetylated ManLev of 9 μM or higher in the medium. At concentrations below 300 nM, there was no detectable antibody-mediated, complement-dependent lysis of Jurkat cells. These results are consistent with data presented in this paper and previously that the surface density of the unnatural sialosides is controlled by the amount of unnatural mannosamine made available to the cell [21]. At exogenous concentrations of acetylated ManLev above 9 μM, the antigen induced on the cells was apparently in excess of that needed to recruit sufficient immunoglobulin

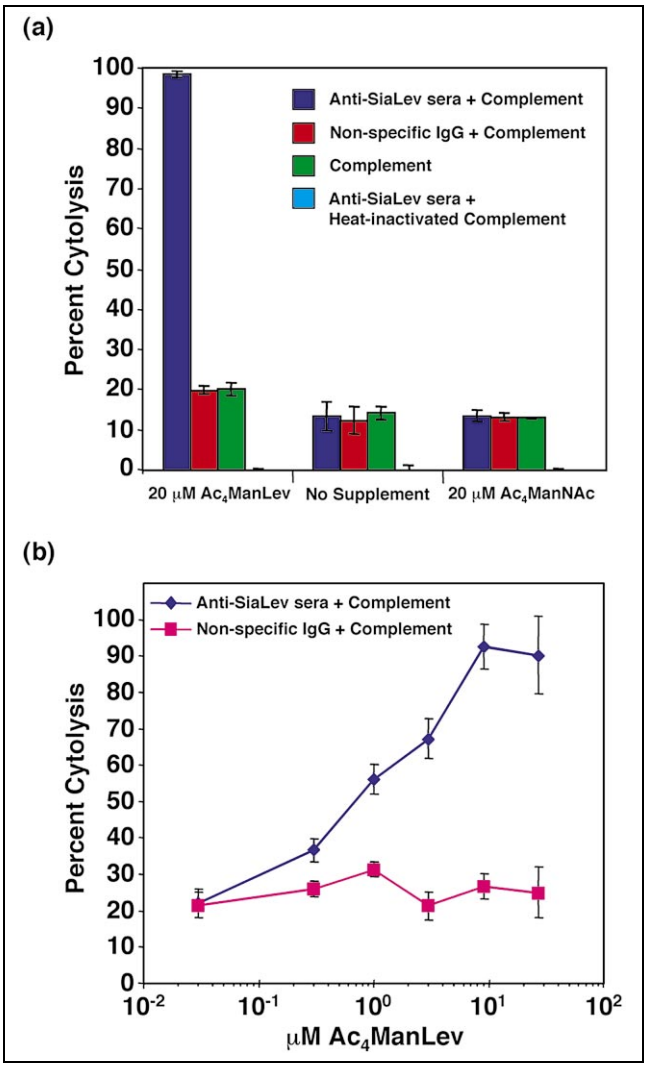

Fig. 6. Antibody-dependent, complement-mediated lysis of SiaLev-expressing cells. (a) Jurkat cells were cultured with 20 μM acetylated ManLev (Ac_4ManLev), 20 μM acetylated ManNAc (Ac_4ManNAc), or with no supplement, then treated with: SiaLev antiserum and 2.5% rabbit serum complement (dark blue), non-specific rabbit IgG and 2.5% rabbit serum complement (red), 2.5% rabbit serum complement only (green), or SiaLev antiserum and 2.5% heat-inactivated rabbit serum complement (light blue). Percent cytolysis was measured as described in Section 4. (b) Jurkat cells were cultured with varying concentrations of acetylated ManLev (0.03–30 μM) over 3 days, then treated with either SiaLev antiserum and 2.5% rabbit serum complement or non-specific rabbit IgG and 2.5% rabbit serum complement. Values for percent cytolysis in both panels a and b are the average of triplicate experiments ± S.D.

for complement activation. Thus, the ability to vary the SiaLev density on target cells provides a mechanism for modulating immunogenicity using unnatural metabolic precursors.

The specificity of the immune response for tumor cell surfaces may be increased if an animal is vaccinated with the SiaLev epitope as a part of a tumor-associated carbohydrate antigen. For example, vaccination with a carbohydrate antigen which incorporates SiaLev as a part of a larger carbohydrate structure should induce an immune

response against the antigen containing SiaLev. Upon administration of acetylated ManLev, the expression of the SiaLev-bearing tumor antigen would be induced, directing immune recognition of the tumor cells expressing the neoantigen. Recently, Liu and coworkers have reported a related strategy to target leukemic cells expressing polysialic acid for immune destruction [43]. Notably, administration of a monoclonal antibody which recognizes poly-*N*-propanoyl sialic acid prevented metastasis of a leukemic cell line when the mice were treated with *N*-propanoylmannosamine, a biosynthetic precursor of poly-*N*-propanoyl sialic acid.

3. Significance

While tumor cells are often characterized by unusual cell surface oligosaccharides, these modifications usually fail to stimulate an immune response against the cell. In order to effect such a response, immune self-tolerance to structures presented on the surface of cancer cells must be broken. Chemical alteration of tumor antigens has been shown to augment the antigenicity of these structures when they are coupled to an appropriate T-cell antigenic epitope. We have shown that immunization of rabbits with an unnatural sialic acid antigen, SiaLev–KLH, leads to the production of significant titers of antibodies following each boost. The antibodies recognized SiaLev to the exclusion of the naturally occurring *N*-acetyl sialic acid. Cells treated with acetylated ManLev exhibited a strong reaction with the SiaLev antisera derived from the rabbit immunization, the extent of which was dependent on the dose of exogenous acetylated ManLev and the amount of immunoglobulin added to cells. The antibodies were capable of stimulating complement-mediated lysis of cells on which SiaLev expression had been induced. Therefore, this work represents a way of breaking immune tolerance for self-antigens, and may have applications in the immunotherapy of cancer given the presence of sialic acid in many tumor-associated carbohydrate antigens.

4. Materials and methods

4.1. Materials

All reagents used in chemical syntheses were obtained from commercial suppliers and used without further purification unless otherwise noted. BSA, KLH, NaN_3, diethanolamine, glutaraldehyde (EM grade), mouse anti-rabbit Ig (clone RG-16; FITC, alkaline phosphatase conjugates), rabbit IgG, RPMI 1640 medium, DME medium, penicillin/streptomycin solution (P/S), 1,4-diazabicyclo(2.2.2)octane (DABCO), and saponin were from Sigma. Trinitrobenzenesulfonic acid and *p*-nitrophenyl phosphate were from Pierce. Sialyl$\alpha(2\rightarrow3)$*N*-acetyllactosamine–BSA and rabbit serum complement were from Calbiochem. Affigel 102® (4% cross-linked aminoalkyl agarose) was from Bio-Rad. Trypsin–EDTA solution was from Gibco BRL. Fetal calf serum (FCS) was from Hyclone. Cytotox-96™ colorimetric cytotoxicity assay kit was from Promega. Alexa® Fluor™-488-conjugated goat anti-rabbit IgG (H+L) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes. *p*-Formaldehyde (16% solution, EM grade) was purchased from Electron Microscopy Sciences. NMR spectra were recorded on Bruker AMX-300 or 400 spectrometers, mass spectral data were obtained at the University of California, Berkeley Mass Spectrometry Facility, immunofluorescent images were obtained on a Zeiss 510 laser scanning confocal microscope and interpreted using Zeiss LSM 5 software, and flow cytometry was performed on a Coulter EPICS™ XL-MCL analytical flow cytometer. Immunizations, animal maintenance, and serum collection was performed according to standard company protocol by Covance Development Services.

4.2. Chemical synthesis

4.2.1. Methyl 5-N-acetamido-4,7,8,9-tetra-O-acetyl-2-O-allyl-N-tert-butoxycarbonyl-3,5-dideoxy-α-D-manno-2-nonulopyranosonate (6)

Compound **5** (0.700 g, 1.28 mmol), DMAP (0.0312 g, 0.256 mmol), and di-*tert*-butyl dicarbonate (0.559 g, 2.56 mmol) were dissolved in anhydrous THF (6 ml). Freshly distilled triethylamine (180 μl, 1.28 mmol) was added via syringe. The solution was stirred for 16 h at room temperature, and then concentrated in vacuo. The resulting syrup was dissolved in 50 ml of CH_2Cl_2, washed with 1 M HCl (2×10 ml), and then saturated $NaHCO_3$ (2×5 ml), and dried over Na_2SO_4. Purification by column chromatography on silica gel eluting with a gradient of hexanes/EtOAc (10:1 to 5:1) afforded **6** as a white foam (0.471 g, 57%): ^{1}H NMR (400 MHz, $CDCl_3$); 4.5:1 mixture of rotational isomers (major): δ 5.78 (m, 1H), 5.31–5.24 (m, 2H), 5.20 (app d, $J=17.2$ Hz, 1H), 5.07 (m, 2H), 4.82 (app t, $J=10.3$ Hz, 1H), 4.63 (app d, $J=10.4$ Hz, 1H), 4.27 (app d, $J=12.5$ Hz, 1H), 4.22 (dd, $J=5.1$, 14.1 Hz, 1H), 3.99 (dd, $J=5.3$, 12.4 Hz, 1H), 3.82 (dd, $J=4.6$, 11.6 Hz, 1H), 3.70 (s, 3H), 2.67 (dd, $J=4.8$, 12.7 Hz, 1H), 2.28 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H), 1.97 (m, 1H), 1.95 (s, 3H), 1.88 (s, 3H), 1.49 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.6, 170.4, 170.0, 169.8, 168.0, 167.5, 151.7, 133.5, 116.9, 98.3, 84.4, 71.0, 68.5, 66.7, 66.5, 65.5, 61.8, 52.4, 52.2, 39.0, 27.7, 26.5, 21.0, 20.8, 20.7, 20.5; HRMS (FAB+) 638.2648 ($M+Li^+$, 638.2636 calc. for $C_{28}H_{41}NO_{15}Li$).

4.2.2. Methyl 2-O-allyl-5-N-tert-butoxycarbonyl-3,5-dideoxy-α-D-manno-2-nonulopyranosonate (7)

A solution of compound **6** (0.471 g, 0.746 mmol) in MeOH (25 ml) was treated with a 1 M solution of NaOMe in MeOH (0.5 ml, 0.5 mmol). The solution was stirred overnight, neutralized with Amberlite IRC-50 (H^+ form), and concentrated to yield **7** (0.291 g, 93%): ^{1}H NMR (300 MHz, CD_3OD) δ 5.87 (m, 1H), 5.25 (dd, $J=1.7$, 17.2 Hz, 1H), 5.13 (dd, $J=1.5$, 10.4 Hz, 1H), 4.30 (m, 1H), 3.98 (m, 1H), 3.79–3.89 (m, 5H), 3.47–3.67 (m, 5H), 2.70 (dd, $J=4.6$, 12.9 Hz, 1H), 1.76 (app t, $J=12.3$ Hz, 1H), 1.46 (s,

9H); ^{13}C NMR (100 MHz, CD_3OD) δ 171.2, 159.6, 135.6, 117.1, 100.0, 81.0, 75.4, 72.7, 70.4, 68.6, 66.3, 64.9, 54.8, 53.5, 41.9, 28.8; HRMS (FAB+) 428.2121 ($M+Li^+$, 428.2108 calc. for $C_{18}H_{31}NO_{10}Li$).

4.2.3. *Methyl 2-O-allyl-5-N-(4-oxo-pentanoyl)-3,5-dideoxy-α-D-manno-2-nonulopyranosonate (**9**)*

Carbamate **7** (0.526 g, 1.25 mmol) was dissolved in CH_2Cl_2 (10 ml) and TFA (1 ml) was added dropwise with stirring. The solution was stirred for 1 h at room temperature, then concentrated in vacuo. Trituration with ether (10 ml) provided a white solid which was dissolved in methanol (5 ml) and treated with sodium methoxide in methanol (1 M, 1.25 ml, 1.25 mmol). After stirring for 15 min, carbonic anhydride **8** [21] (1.4 mmol) in THF (4 ml) was added dropwise. The resultant yellow solution was stirred overnight, and then concentrated. Silica gel chromatography ($CHCl_3$/MeOH, 50:1 to 10:1) provided a yellow foam which was further purified by reversed-phase HPLC (CH_3CN in H_2O, 0–20% over 45 min) to afford compound **9** as a white solid (98.6 mg, 40% based on **7**): ^{1}H NMR (400 MHz, D_2O) δ 5.85 (m, 1H), 5.26 (dd, J = 1.5, 17.3 Hz, 1H), 5.19 (app d, J = 10.4 Hz, 1H), 4.26 (dd, J = 5.7, 12.3 Hz, 1H), 4.01 (dd, J = 6.1, 12.3 Hz, 1H), 3.82–3.77 (m, 7H), 3.71 (m, 1H), 3.60 (m, 1H), 3.49 (app d, J = 8.8 Hz, 1H), 2.83 (m, 2H), 2.67 (dd, J = 4.6, 12.8 Hz, 1H), 2.47 (m, 2H), 2.16 (s, 3H), 1.76 (app t, J = 12.3 Hz, 1H; ^{13}C NMR (100 MHz, D_2O) δ 213.9, 176.2, 170.1, 133.2, 118.61, 98.9, 73.0, 70.7, 68.4, 67.1, 65.9, 63.2, 53.4, 51.8, 29.5, 29.2; HRMS (FAB+) 420.1875 ($M+H^+$, 420.1870 calc. for $C_{18}H_{30}NO_{10}$).

4.2.4. *Compound **10***

Allyl glycoside **9** (191.5 mg, 0.457 mmol) was dissolved in aqueous NaOH (1 M, 0.461 ml, 0.461 mmol) and stirred at room temperature for 4 h. A solution of 2-aminoethanethiol hydrochloride (57 mg, 0.50 mmol) in H_2O (0.46 ml) was then added, and the solution was irradiated for 16 h at room temperature with 254 nm light from an 18 W lamp under a N_2 atmosphere. Purification of the reaction mixture by reversed-phase HPLC (acetonitrile in water, 0–20% over 45 min) provided amine **10** (131.6 mg, 60% based on **9**): ^{1}H NMR (400 MHz, D_2O) δ 3.84–3.75 (m, 3H), 3.71 (app d, J = 9.9 Hz, 1H), 3.66–3.56 (m, 3H), 3.50 (m, 2H), 3.17 (t, J = 6.5 Hz, 2H), 2.81 (m, 4H), 2.67 (dd, J = 4.7, 12.6 Hz, 1H), 2.60 (app t, J = 8.0 Hz, 2H), 2.47 (m, 2H), 2.16 (s, 3H), 1.80 (m, 2H), 1.58 (app t, J = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 213.9, 176.3, 173.6, 100.7, 72.7, 71.9, 68.3, 68.1, 63.1, 62.7, 51.9, 40.5, 38.3, 38.1, 29.4, 29.2, 28.9, 28.2, 27.3; HRMS (FAB+) 483.2003 ($M+H^+$, 483.2012 calc. for $C_{19}H_{35}N_2O_{10}S$).

4.3. *Preparation of neoglycoconjugates*

4.3.1. *SiaLev–KLH (**3**)*

Amine **10** (0.030 g, 0.062 mmol) was dissolved in H_2O (600 μl). Solid Na_2CO_3 (8.5 mg, 0.068 mmol) and $NaHCO_3$ (10.4 mg, 0.124 mmol) were added, adjusting the solution to pH 9. A solution of thiophosgene (5.9 μl, 0.078 mmol) in $CHCl_3$ (300 μl) was then added to the vigorously stirred solution of compound **10** at room temperature. After 2.5 h, amine **10** was essentially consumed according to TLC (butanol/acetic acid/water; 5:3:2, **10**: R_f = 0.6, product: R_f = 0.8) and negative ninhydrin staining. The mixture was concentrated and the crude product was reconstituted in H_2O for direct use in protein conjugation reactions. An aqueous solution (700 μl) of the isothiocyanate derived from **10** (233 μl, 10 mg, 0.021 mmol) was added to KLH (8 mg) dissolved in 800 μl of 300 mM $NaHCO_3$ buffer (pH 9). The mixture was incubated for 12 h at room temperature and then diluted with 2 ml of PBS and dialyzed against 3×1.5 l of PBS over 24 h. The trinitrobenzene sulfonic acid assay for unconjugated lysine residues [44] revealed that 69% of lysine residues for conjugation were modified by **10** using this procedure. On average each KLH molecule was modified with 180 copies of the hapten.

4.3.2. *SiaLev–BSA*

The same procedure used with KLH was applied to BSA, resulting in the modification of approximately 57% of the available lysines. On average, each BSA molecule was modified with 19 copies of SiaLev.

4.3.3. *SiaLev–agarose*

Aminoalkyl agarose (5 ml, 4% cross-linked, with a reactive amine concentration of 16.4 μmol/ml gel) was equilibrated in 300 mM $NaHCO_3$ (pH 9) buffer. An aqueous solution of **10** (1.03 ml, 114 μmol) that had been activated with thiophosgene was added to the suspension. The suspension was agitated by gentle rocking over 16 h. The resin was then washed with 100 ml of PBS and used for affinity purification.

4.3.4. *Sialic acid–agarose*

The same procedure used to prepare SiaLev–agarose was applied to sialic acid–agarose.

4.3.5. *Affinity purification of polyclonal sera*

Whole serum (5 ml) was diluted 1:10 with buffer 1 (10 mM Tris: pH 7.5), and precleared by passage through a Sia–agarose column (1.5 ml bed volume) three times over 3 h. The flow-through was then applied to a SiaLev–agarose column (1 ml bed volume) three times over 3 h. The column was washed with 20 bed volumes of buffer 1, followed by 20 bed volumes of buffer 1+500 mM NaCl. The SiaLev column was then eluted with 15 bed volumes of the following: 100 mM glycine (pH 2.5), 10 mM Tris (pH 8.8), and 100 mM triethylamine (pH 11.5, prepared from freshly distilled triethylamine). All eluates were collected in tubes containing 1.5 bed volumes of 1 M Tris (pH 8.0). Fractions containing eluate were combined, concentrated, and dialyzed repeatedly against PBS containing 0.02% NaN_3.

4.3.6. *Determination of antibody titers by ELISA*

Microtiter plate wells (Dynex Immulon II-HB) were coated with 50 μg/ml solutions of SiaLev–BSA, or SiaLacNAc–BSA (50 μl/well) in PBS for 4 h at room temperature, then blocked with buffer 2 (PBS+3% BSA+0.02% NaN_3) overnight at 4°C. The plates were then washed twice with PBS, and serial dilutions of

antisera in buffer 2 were added to duplicate wells (50 µl/well), and incubated for 2 h at room temperature. The plate was washed twice with PBS, followed by three washes with 25 mM Tris-buffered saline (pH 7.5) (TBS). A secondary antibody (mouse anti-rabbit Ig: clone RG-16)-alkaline phosphatase conjugate diluted 1:1000 in TBS+3% BSA+0.02% NaN_3 was added to each well (50 µl/well), and incubated at room temperature for 2 h. The plate was washed four times with TBS, once with buffer 3 (10 mM diethanolamine pH 9.6+0.5 mM $MgCl_2$), and a 0.5 mM solution of *p*-nitrophenyl phosphate in buffer 3 was added (100 µl/well). The absorbance at 405 nm was then measured. The titer of antibody in each batch of serum was defined as the dilution of serum which corresponded to the absorbance at half the maximum of the titration curve.

4.3.7. Tissue culture/cell culture conditions

Unless otherwise specified, Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (P/S); HeLa cells were cultured in DME medium supplemented with 10% FCS, and P/S. In all cases cells were cultured under a humidified atmosphere containing 5% CO_2 at 37°C. Jurkat cells were seeded at cell densities of 125 000 to 250 000 cells/ml in 10–15 ml of medium in T-25 flasks and cultured in suspension. HeLa cells were cultured in 110 cm^2 tissue culture polystyrene dishes, seeded at a density of 9000 cells/cm^2 in 10 ml of medium, and cultured as adherent monolayers. For flow cytometry experiments, adherent HeLa cells were trypsinized with trypsin–EDTA, and suspended in DME medium prior to antiserum treatment. When appropriate, cells were cultured in the presence of acetylated ManLev, ManNAc, or ManPent by adding the appropriate volume of a 50–100 mM stock solution of the compound dissolved in 95% ethanol to the medium.

4.3.8. Flow cytometry assay for cell surface binding of SiaLev antiserum

All steps were performed at 4°C. Jurkat or HeLa cells ($10–20\times10^6$) were washed twice with 10 ml of buffer 4 (PBS, pH 7.4, 2% FCS, 0.02% NaN_3) and resuspended at a density of 1.5×10^6cells/ml. The cell suspension was transferred in 200 µl aliquots (3×10^5 cells) to the appropriate well in a 96 well V-bottomed microtiter plate. The cells were then isolated by centrifugation ($1200\times g$, 4 min), and resuspended in buffer 4 (100 µl) containing the appropriate type and dilution of serum. The cells were incubated for 1 h then washed with buffer 4 (3×200 µl), and resuspended in 150 µl of buffer 4. FITC-labeled mouse anti-rabbit Ig (clone RG-16) diluted 1:100 in buffer 4 was added to each of the wells in 50 µl aliquots. The cells were incubated in the dark for 1 h. The cells were then washed with buffer 4 (3×200 µl), resuspended in 400 µl of buffer 4, and analyzed by flow cytometry.

4.3.9. Immunofluorescence analysis of intracellular SiaLev distribution

A suspension of HeLa cells (1×10^6/ml) produced as described above that had been cultured for 2 days in medium supplemented with or without acetylated sugars was added in 200 µl aliquots (200 000 cells/well) to 9.6 cm^2 wells which contained glass coverslips (22 mm/side) and 5 ml growth medium supplemented with 30 µM of the appropriate acetylated carbohydrate. After 2 days of growth, the coverslips were washed with PBS (3×5 ml) at 37°C. Cells were fixed on the coverslips at 37°C for 20 min then at 4°C for 15 h in a buffer at pH 7.6 containing the following: 2% *p*-formaldehyde, 0.1% glutaraldehyde, 0.1% saponin, and 100 mM KCl, 3 mM $MgCl_2$, 8 mM HEPES, 150 mM sucrose. Following fixation, each coverslip was washed three times with PBS, pH 7.4, then blocked for 20 min with buffer 2. The coverslips were then treated with 500 µl of a 2.5 µg/ml solution of affinity-purified SiaLev antiserum or non-specific rabbit IgG as a control in buffer 2 for 1.25 h, at room temperature with gentle rocking. The coverslips were then washed three times each with 1.5 ml of buffer 2 (10 min incubation/wash). Alexa-488-labeled goat anti-rabbit IgG (H+L) diluted in buffer 2 was applied to each coverslip (500 µl, 2 µg/ml) and incubated in the dark at room temperature with gentle rocking for 1.25 h. The coverslips were then washed four times with 1.5 ml of buffer 2, followed by two 1.5 ml washes with PBS. Coverslips were then inverted and mounted on a drop of mounting medium containing DAPI (90% glycerol, 10% Tris pH 7.5, 1 µg/ml DAPI, and DABCO). Slides were analyzed under oil immersion at $1000\times$. Images were obtained by dual track imaging using the 488 nm line of a Kr-Ar laser for excitation with 505 nm LP optics on track 1 and the 364 nm line of an argon ion laser with 435–485 BP optics on track 2. Images shown are 2 µm thick single optical sections through the center of the nuclei and are the result of merging the data from tracks 1 and 2 which are pseudo-colored green and blue, respectively, in the composite images.

4.3.10. Complement-mediated lysis

Jurkat cells were washed twice with buffer 5 (PBS+0.5 mM $MgCl_2$+0.15 mM $CaCl_2$+5% FCS) and suspended at a density of 1.5×10^6 cells/ml in buffer 5. Cells were then transferred into the appropriate wells (160 µl/well) of a 96 well V-bottomed microtiter plate which also contained 250 ng of affinity-purified anti-SiaLev serum diluted in 40 µl of buffer 5. Cells were incubated at room temperature for 30 min. Rabbit serum complement diluted 1:4 in buffer 5 (50 µl) was added to each well. For control experiments, the rabbit serum complement was inactivated by heating at 56°C for 1 h prior to addition to the cell suspension. The cell suspension was incubated for 37°C for 1 h, then centrifuged at $250\times g$ for 5 min. An aliquot of supernatant (50 µl) from each well was transferred to the identically labeled well of a 96 well flat-bottomed microtiter plate. The amount of lactate dehydrogenase (LDH) activity present in each well was quantified using the diaphorase/tetrazolium development solution in Promega's Cytotox-96 cytotoxicity kit according to the manufacturer's instructions, followed by measuring the absorbance of each well at 490 nm. The percent lysis was calculated using absorbance values (average of triplicate samples) according to the following formula: % Cytolysis = (experimental lysis minus spontaneous lysis)/(maximal lysis minus spontaneous lysis).

Spontaneous lysis was defined as the LDH activity released by cells treated only with buffer 5 during the course of the assay.

Maximal lysis was defined as the LDH activity of wells in which cells were treated with 50 μl of 5% Triton X-100 lysis buffer (Promega) as a substitute for complement.

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