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Genetically Altered Mice with Different Sialyltransferase Deficiencies Show Tissue-specific Alterations in Sialylation and Sialic Acid 9-*O*-Acetylation*[S]

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Glycan chains on glycoconjugates traversing the Golgi apparatus are often terminated by sialic acid residues, which can also be 9-*O*-acetylated. This process involves competition between multiple Golgi enzymes. Expression levels of Golgi enzyme mRNAs do not always correlate with enzyme activity, which in turn cannot accurately predict glycan sequences found on cell surfaces. Here we examine the cell type-specific expression of terminal glycans in tissues of normal mice in comparison with animals deficient in ST6Gal-I (transfers α2–6-linked sialic acid to Galβ1–4GlcNAc) or ST3Gal-I (transfers α2–3-linked sialic acid to Galβ1–3GalNAc). Tissues of ST6Gal-I null mice showed minimal binding of an α2–6-sialic acid-specific lectin, indicating that no other enzyme generates Siaα2–6Galβ1–4GlcNAc and that Siaα2–6GalNAc (sialyl-Tn) is rare in mice. However, exposed Galβ1–4GlcNAc termini were only moderately increased, indicating that these can be partially capped by other enzymes. Indeed, Galα1–3Galβ1–4GlcNAc and Fucα1–2Galβ1–4GlcNAc termini were enhanced in some tissues. Many tissues of ST3Gal-I null animals showed increases in Galβ1–3GalNAc termini, and some increases in poly-*N*-acetyllactosamines. However, overall expression of α2–3-linked sialic acid was selectively reduced only in a few instances, indicating that other ST3Gal enzymes can generate this linkage in most tissues. Highly selective losses of 9-*O*-acetylation of sialic acid residues were also observed, with ST6Gal-I deficiency causing loss on endothelium and ST3Gal-I deficiency giving a marked decrease on $CD4^+$ lymphocytes. These data demonstrate selective regulation of sialylation and 9-*O*-acetylation, point to cell types with potential physiological defects in null animals, and show *in vivo* evidence for competition between Golgi enzymes.

The outer surfaces of all eukaryotic cells are covered with a dense and complex array of glycosylated molecules (1). More than 200 gene products (mostly glycosyltransferases) are involved in generating the developmentally regulated and tissue-specific glycosylation characteristic of each cell type in mammals (2, 3). The biosynthesis of glycan chains takes place mostly in compartments of the endoplasmic reticulum-Golgi pathway in stepwise reactions involving specific sugar nucleotide transporters, glycosyltransferases, glycosidases, and other sugar-modifying enzymes (4–7). Expression of some of these enzymes is altered in embryogenesis (8), cancer (9, 10), injury, and inflammation (11, 12). Indeed, altered glycosylation is a universal feature of cancer cells, and certain glycan structures are well known markers for tumor progression (9, 10).

N- and *O*-glycans and glycosphingolipids are often terminated by the addition of sialic acids, a family of nine carbon carboxylated monosaccharides (13–16). Because of their terminal location and negative charge, sialic acids have the potential to inhibit many intermolecular and intercellular interactions (17). The structural diversity of sialic acids (16) can also determine or modify recognition by antibodies, by a variety of endogenous sialic acid-binding lectins, as well as by microbial agglutinins, toxins, and adhesins (18, 19). Sialic acids are involved in many other vertebrate functions, including cell-cell interactions in processes such as the trafficking of blood cells during inflammation (12), the control of neuronal plasticity (17), and the interactions of tumor cells during the metastatic process (9, 10).

Sialic acid residues can be attached to the underlying sugar chain via α2–3, α2–6, or α2–8 linkages. One or more sialyltransferases (STs)[1] have been characterized and cloned for each linkage type (20–24). The α2–3 sialic acid linkages to Gal residues seem to be the most widely expressed; these are followed next in frequency by the α2–6-linkage of sialic acid to Gal or GalNAc. Some of these sialic acid residues can also be modified by the addition of *O*-acetyl esters, especially at the 9-position. These ester groups appear to be added by the action of a family of linkage-specific *O*-acetyltransferases that have yet to be cloned (16, 25–30). The addition of *O*-acetyl groups can substantially modify the role of sialic acids in a variety of biological processes (13–16, 31).

To help elucidate the functions of α2–3- and α2–6-linked sialic acids in an intact vertebrate animal system, the ST genes responsible for producing some of these linkages have been inactivated in the mouse. ST6Gal-I generates an α2–6 linkage of sialic acid to underlying *N*-acetyllactosamine (32, 33). The resulting trisaccharide structure (Siaα2–6Galβ1–4GlcNAc) is

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains a table of lectin reactivities on mouse tissues.

[1] The abbreviations used are: ST, sialyltransferase; Sia, sialic acid; ST6Gal-I, Galβ1–4GlcNAc:CMP-sialic acid ST; ST3Gal-I, Galβ1–3GalNAc:CMP-sialic acid ST; SNA, *Sambucus nigra* agglutinin; ECA, *Erythrina crystagalli* agglutinin; PNA, *Arachis hypogea* peanut agglutinin; MAH, *Maackia amurensis* hemagglutinin; MAL, *Maackia amurensis* lectin; GSL-1, *Griffonia simplicifolia* lectin-1; TL, *Lysopersicon esculentum* tomato lectin; UEA, *Ulex europaes* agglutinin; CHE-FcD, influenza C hemagglutinin esterase IgG-Fc fusion protein treated with DFP, diisopropyl fluorophosphate; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorter.

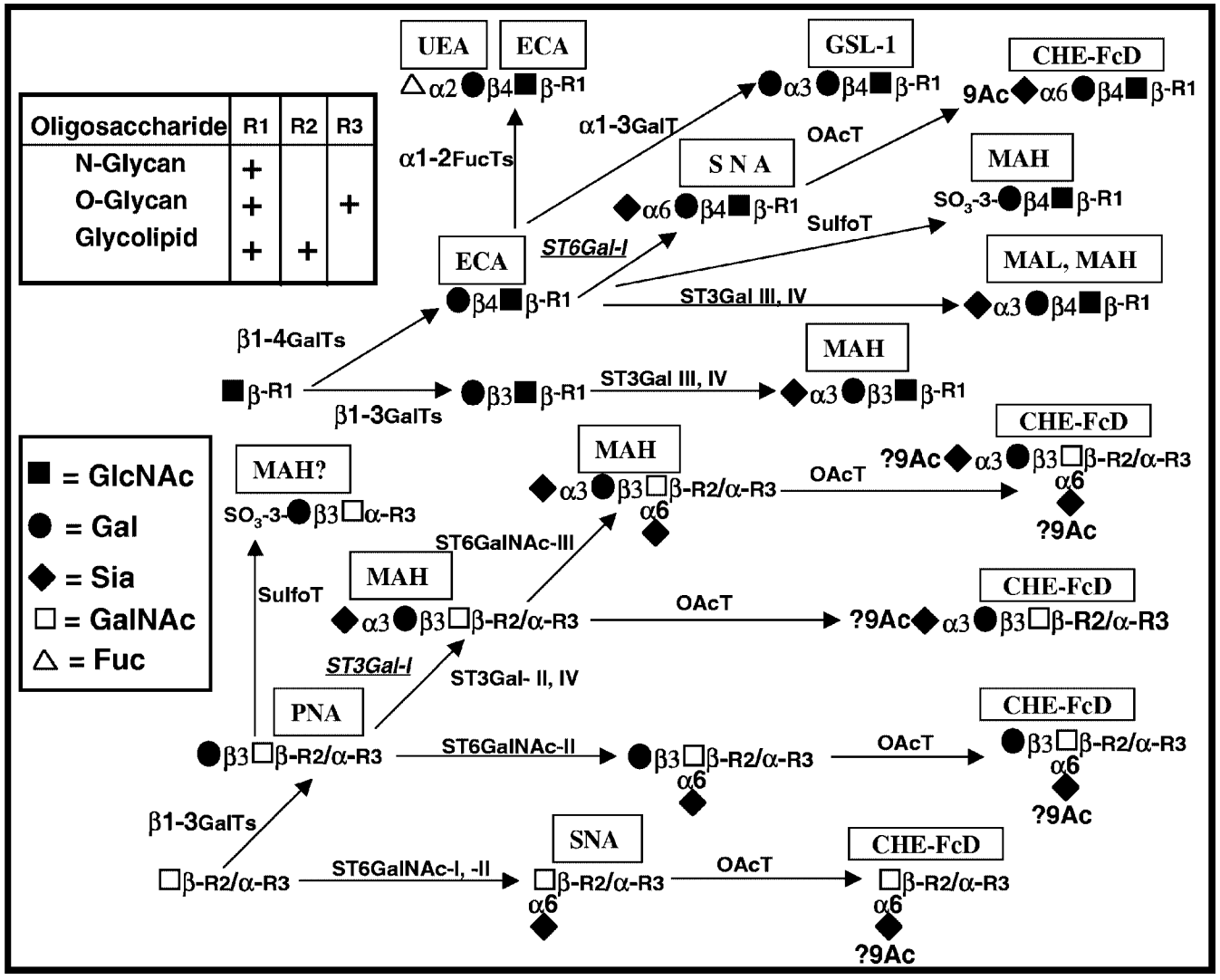

FIG. 1. **Biosynthetic pathways for some common sialylated glycans and specificity of the probes used in this study.** Sialic acids are commonly attached to underlying glycan chains in α2–3 or α2–6 linkage, usually terminating chain extension. Pathways for chain extension and sialylation of some common sialylated glycans are shown. Biosynthesis of these specific linkages is dictated by specific STs, and the precursor or product glycan structures can be recognized by various lectin probes. Lectins recognizing specific glycans are indicated in *boxes above* the corresponding structure(s). Sialic acids can be further modified by 9-*O*-acetyl groups, which can be detected using the CHE-FcD probe. The *question marks* denote instances in which the location of the 9-*O*-acetyl group is not yet certain. *Sambucus nigra* agglutinin (*SNA*) binds to sialic acids in an α2–6 linkage to the underlying Gal or GalNAc (57–59), regardless of whether it is in the *N*-glycolyl form and/or is 9-*O*-acetylated (43). *Erythrina crystagalli* agglutinin (*ECA*) binds primarily to terminal Galβ1–4GlcNAc (60, 61) as long as it is not capped with any unit other than Fucα1–2 (44). *Maackia amurensis* lectin (*MAL*, called MAL-I by Vector Laboratories) specifically recognizes Galβ1–4GlcNAc units and binding is enhanced by α2–3-linked sialic acid capping (40, 62–65). *Maackia amurensis* hemagglutinin (*MAH*, called MAL-II by Vector Laboratories) recognizes α2–3-linked sialic acids (40, 43, 62–64) or 3-*O*-sulfate esters (45) on galactose termini. Peanut agglutinin (*PNA, Arachis hypogea* agglutinin) binds preferentially to uncapped Galβ1–3GalNAc (so-called "T-antigen") (66–68) but can also bind weakly to high densities of uncapped Galβ1–4GlcNAc termini. *Griffonia simplicifolia* lectin-1 (*GSL-1*) binds terminal α1–3-linked Gal residues. Tomato lectin (*TL*) recognizes polylactosamine extensions. *Ulex europaes* agglutinin (*UEA*) binds terminal α1–2-linked fucose residues. Influenza CHE-FcD binds specifically to 9-*O*-acetylated sialic acids regardless of the underlying linkage. This is a recombinant probe containing the extracellular domain of the Influenza C virion's hemagglutinin esterase (*CHE*) protein fused to the Fc region of human IgG_1 (41). Treatment of this chimeric molecule (*CHE-Fc*) with diisopropylfluorophosphate selectively eliminates its esterase activity while preserving and enhancing its binding property (*CHE-FcD*) (41).

the ligand for the Siglec-2/CD22 lectin of B-lymphocytes (34). The absence of ST6Gal-I in mice results in an immunodeficiency characterized by attenuated B lymphocyte function and failure to generate high titers of antibodies in response to T-dependent and T-independent antigens (35). Upon immunization or antigen receptor cross-linking, ST6Gal-I-deficient B cells exhibit reduced calcium fluxes and diminished phosphotyrosine accumulation on key cellular signal transducers (35). ST3Gal-I generates an α2–3 sialic acid linkage on the Galβ1–3GalNAc disaccharide, primarily on *O*-glycan chains (36). The absence of ST3Gal-I in mice results in a marked deficiency of mature cytotoxic $CD8^+$ T-lymphocytes, apparently via enhanced apoptosis (37, 38). Although these are the most prominent pathological consequences of these enzyme deficiencies, it remains to be seen whether there are deleterious effects in other cell types in which these enzymes are normally expressed.

Plant lectins have been powerful tools used to explore glycan structures (39, 40). Because of the specificity that each lectin has toward particular structures, even isomeric glycans with identical sugar compositions can be distinguished. Here we have used various plant lectins in flow cytometry and immunohistochemistry assays to characterize changes resulting from genetic inactivation of *ST3Gal-I* and *ST6Gal-I* genes in mice. We also used a recombinant chimeric soluble form of the influenza C hemagglutinin esterase (41) to detect changes in

TABLE I
Specificity of lectins used for immunohistochemistry or flow cytometry
See text and legend to Fig. 1 for discussion of Table I.

Lectin	Binding specificity		Controls
	Terminal	Underlying saccharide	
SNA	Siaα2–6 required	Gal or GalNAc	Mild periodate abolishes (if Sia is not 9-*O*-acetylated)
PNA	Sia blocks binding	Galβ1–3GalNAc	0.1 M lactose
		Galβ1–4GlcNAc (weak)	Secondary reagent alone
MAH	Siaα2–3 or 3-*O*-sulfate	Galβ1-	Secondary reagent alone
ECA	Sia blocks Fucα1–2 OK	Galβ1–4GlcNAc	0.1 M lactose inhibits
			Secondary reagent alone
GSL-1	Galα1–3 required	Galβ1–4GlcNAc	0.1 M mellibiose inhibits
			Secondary reagent alone
TL	Can be varied	$(Gal\beta1–4GlcNAc)_n$	Chitobiose inhibits
			Secondary reagent alone
UEA	Fucα1–2 required	Galβ1-(3)4GlcNAc	Secondary reagent alone
MAL	Siaα2–3 or none	Galβ1–4GlcNAc	Secondary reagent alone
CHE-FcD	9-*O*-acetyl Sia	Can be varied	CHE-Fc (untreated with DFP)

TABLE II
Summary of major lectin reactivity differences observed in organs of ST3Gal-I null and ST6-Gal null mice in comparison with wild-type animals
For details, see Figs. 2–6 and the table in the "Supplemental Material."

Lectin binding	ST6Gal-I null tissues	ST3Gal-I null tissues
SNA	Absent in all except some cartilage in embryos and some smooth muscle in adults	No change
PNA	Some increase in spleen	Diffusely increased in all organs
MAH	Some increase in spleen only	Decreased in spleen, thymus, and kidney and absent in adrenal
ECA	Increased in spleen	No change
GSL-1	Increased in spleen	No change
TL	No change	Increased in spleen
UEA	Small increase in spleen	No change
CHE-FcD	Absent in blood vessel endothelium of all organs	Decreased binding to lymphocytes in lymph node T-cell zones

sialic acid 9-*O*-acetylation. In exploring these issues we were particularly interested in determining the outcome of these deficiencies on the potentially competitive glycosyltransferase reactions thought to take place in the late Golgi apparatus, *e.g.* with other STs and certain α-fucosyltransferases, and α-galactosyltransferases. It is also of interest to know whether these competing transferases always take over use of the acceptor chains or whether some are left exposed. Furthermore, do any resulting losses or changes in sialyl linkages affect sialic acid *O*-acetylation? Do any of the changes seen take place in a tissue-specific manner? In the course of exploring such questions we generated, for the first time, a base-line description of the patterns of expression of various glycan termini in the organs and tissues of the normal laboratory mouse, as well as uncovered clues to other potential biological consequences of ST3Gal-I and ST6Gal-I deficiency.

EXPERIMENTAL PROCEDURES

Generation of ST3G-I Null Mice and ST6Gal-I Null Mice— ST3G-I null and ST6Gal-I null mice were generated as described elsewhere (35, 42). Genomic clones of the *ST6Gal-I* and *ST3Gal-I* genes were used in constructing targeting vectors that incorporate Cre/loxP recombination sites. The selected mutations deleted critical exons necessary for protein translation and catalytic function.

Histology and Immunohistochemistry—Organs from 4–6-week-old wild-type and null animals were harvested and processed immediately for frozen or paraffin sections. Tissues to be frozen were surrounded with OCT (Optimum Cutting Temperature, Fisher Scientific) compound in freezable plastic molds, and immersed in a slurry of dry ice/2-methyl butane. The frozen blocks were stored in labeled bags at −70 °C and removed when necessary for frozen sectioning and staining. Tissues to be paraffin-embedded were fixed in 10% buffered formalin for 24 h before being transferred to 70% ethanol for the subsequent dehydration and paraffin embedding process. Day 16.5 embryos from wild-type and null animals were processed similarly for frozen or paraffin sagittal sections.

Sections from wild-type and null mice were placed side by side on the same slide, so that both would be treated identically during the staining processes. This placement also allowed sequential visualization and comparison of wild-type and null organs in a systematic fashion. All tissue sections were also stained with hematoxylin and eosin to facilitate the review of the morphology and/or pathology when adjacent sections were evaluated by immunostaining. All immunostaining experiments included both negative and positive staining controls (see Table I). In addition to using the secondary reagent alone as a negative control, we sometimes incubated serial sections with an excess of appropriate saccharide to inhibit binding of the lectin. An additional control for the SNA included preincubation of an adjacent section with mild periodate (which eliminates recognition by truncating the sialic acid side chain if there is no 9-*O*-acetyl group present).

Biotinylated lectins or lectins directly conjugated to enzyme or fluorescent tags were purchased from Vector Laboratories (Burlingame, CA) for use in the immunostaining experiments. Alkaline phosphatase conjugates were generally avoided due to the presence of endogenous alkaline phosphatase in frozen sections of some tissues, which can confound interpretation. Lectins were diluted to working concentrations in 1% bovine serum albumin in phosphate-buffered saline, pH 7.1 (1% BSA/PBS) to decrease nonspecific binding to matrix components. Binding of horseradish peroxidase conjugates was visualized with either diaminobenzidine or 3–3-aminoethylcarbazole (AEC, Vector Laboratories). Following enzyme-labeled detection, the morphology of the underlying tissue sections was revealed by staining nuclei using aqueous Mayer's hematoxylin. The sections were then prepared for viewing under the microscope by cover-slipping in aqueous mounting media.

Although the staining patterns were generally similar in paraffin sections, the intensity was sometimes remarkably decreased when compared with frozen sections, and we therefore proceeded with the complete study using only the latter. Frozen sections of spleen, thymus, and lymph nodes were always fixed in acetone for 10 min before proceeding. The rest of the organs were fixed in 10% buffered formalin for 30 min

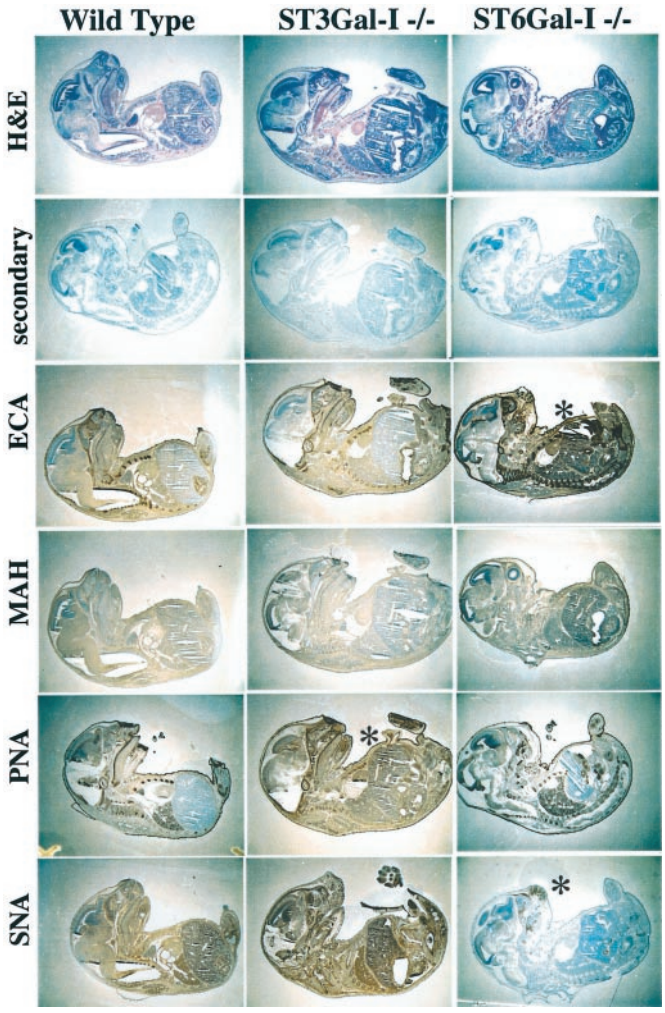

FIG. 2. **Lectin staining of saggital sections of day 16.5 embryos.** Binding of biotinylated SNA, PNA, MAH, or ECA to frozen sections of embryos from wild-type, ST6Gal-I null, and ST3Gal-I null mice was detected using HRP-streptavidin as described under "Experimental Procedures." The *panels* labeled "*secondary*" were exposed to the secondary reagent only. The *asterisks* indicate the sections on which major changes were seen.

before a buffer rinse and subsequent lectin staining. Tissues that contained endogenous biotin were blocked with 0.1% avidin for 10 min followed by buffer rinses and incubation in 0.01% biotin for 10 min and additional buffer rinses before being treated with 1% bovine serum albumin to block nonspecific binding.

Organs from at least six different sets of animals were compared for consistency in the observed patterns. Similar areas from the different organs from the wild-type animals and the null animals were compared to acquire data. For example, if an area of kidney cortex near the arcuate arteries was being evaluated, a similar area in both the wild-type and the null animal kidney was evaluated. For base-line information, all organs were initially characterized as to typical staining patterns of specific areas in particular organs with each of the lectins.

Flow Cytometry Analysis—Single cell suspensions from thymus, spleen, or lymph nodes of 4–6-week-old mice were subjected to red blood cell lysis by ammonium chloride. Peripheral blood mononuclear cells were isolated by a density gradient using Lympholyte. Prior to staining, CHE-FcD (2 μg) was pre-complexed to phycoerythrin-conjugated goat anti-human IgG (1:100) in 100 μl of FACS buffer (1% bovine serum albumin, 0.1% sodium azide in phosphate-buffered saline) on ice for 1 h. Cell staining was performed using 1 million cells in 100 μl of FACS buffer on ice for 20 min (using anti-CD4 or anti-CD8) or 2 h (using CHE-FcD). For double and triple staining, cells were incubated first with pre-complexed CHE-FcD, washed, and then stained with the anti-CD4 and/or CD8 reagent(s). After staining, cells were washed with excess buffer and resuspended in 300 μl of fixation buffer (1% formaldehyde in phosphate-buffered saline). Stained, fixed cells were analyzed on the same day. Fluorescein isothiocyanate- or tricolor-conjugated rat anti-CD4 and anti-CD8 and phycoerythrin-conjugated goat anti-human IgG (Fc-specific) antibodies were from Caltag. Data were acquired using a BD FACScan and analyzed by the CELLQUEST program.

RESULTS AND DISCUSSION

The glycan binding specificities of the lectins and probes used in this study (40) are outlined in Fig. 1, and the specificity of the lectins used for histochemical staining is described in Table I and in the legend to Fig. 1. The major changes seen in the ST3Gal-I or ST6Gal-I null mice are summarized in Table II. A third table (see the Supplemental Material) provides more details about the changes that were seen in specific organs of null animals

Altered Terminal Glycosylation in ST6Gal-I Null Mice—No major histological abnormalities were observed in a survey of hematoxylin and eosin-stained tissues of embryonic or young adult ST6Gal-I null mice (data not shown). The major change seen was a loss of binding to the α 2–6-specific SNA lectin. As shown in Fig. 2, analysis of wild-type day 16.5 embryos showed diffusely positive staining with SNA. In contrast, almost no staining was seen in ST6Gal-I null embryos, except for some weak reaction of cartilage and smooth muscle. This staining was eliminated by prior treatment with mild periodate (not shown), indicating that it was specific. Specific staining with SNA was also seen in many organs and cell types of adult wild-type mice (see Fig. 3 for some examples and Tables II and III for a summary of the findings). In contrast, none of the organs of the adult ST6Gal-I null mice stained with SNA (except for some staining in the smooth muscle of the tracheobronchial tree). Similar results were observed using either enzyme-labeled secondary reagents or the more sensitive fluorescent reagents (Fig. 3).

Taken together, these results indicate that there may be no other enzyme that can produce the same linkage sequence (Siaα2–6Galβ1–4GlcNAc) as the ST6Gal-I enzyme. The data also indicate that Siaα2–6GalNAcα1-*O*-Ser/Thr (the sialyl-Tn epitope, which can also be recognized by SNA) (43) must be very rare in normal mouse tissues. It is possible that the latter structure accounts for the weak staining of cartilage and smooth muscle in the day 16.5 embryos and the tracheobronchial smooth muscle in adults.

The loss of ST6Gal-I activity in the null mice could potentially result in exposure of terminal Galβ1–4GlcNAc units that can be recognized by ECA. If there is no effective competition by other late Golgi enzymes, one would expect to see ECA positivity in all the places where SNA reactivity is lost. However, although there was a moderate general increase in ECA staining in the day 16.5 embryo (Fig. 2), adult animals showed increased ECA staining only within the spleen (Fig. 4) and kidneys (not shown). A moderate increase in PNA binding was also seen in the same areas (Fig. 4), likely due to the weak reactivity of PNA with Galβ1–4GlcNAc units. These data indicate that other competing glycosyltransferases must be effectively utilizing most of the Galβ1–4GlcNAc units that fail to be capped by ST6Gal-I in all the other major cell types throughout the adult body. Staining with MAH (which recognizes the product of α2–3 STs) showed no major increase over the wild-type levels (see Fig. 4). However, increased staining with GSL-1 was seen in several tissues, indicating that the α1–3-galactosyltransferase generating Galα1–3Galβ1–4GlcNAc units has a greater opportunity to act in the absence of ST6Gal-I (see Fig. 5 for an example). An additional possibility is that the Galβ1–4GlcNAc units are capped by α1–2-linked fucose residues, which do not prevent the binding of ECA (44). In keeping with this possibility, some increased staining with UEA was observed in the spleen.

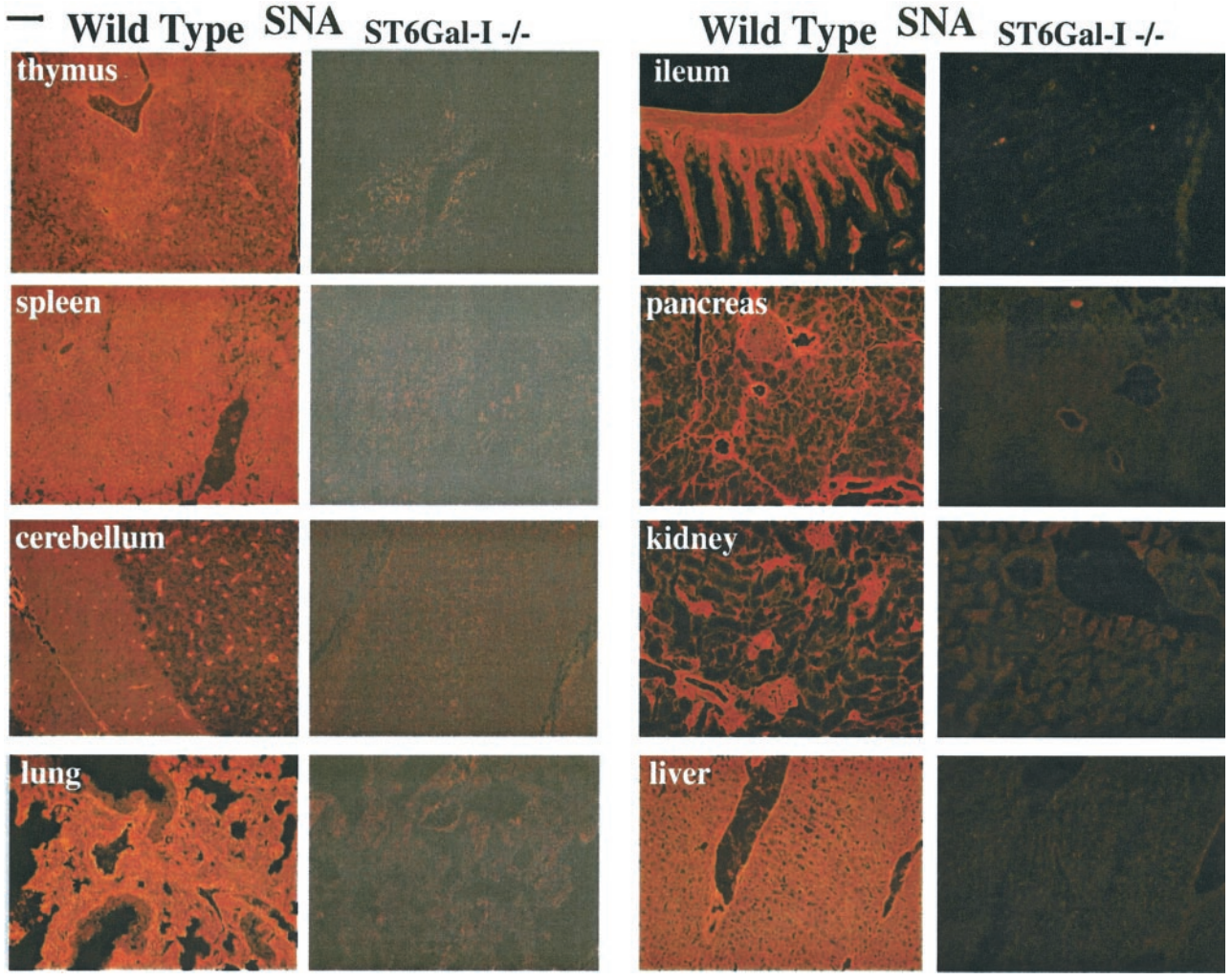

FIG. 3. **Changes seen in multiple organs of ST6Gal-I null animals with SNA fluorescent histochemical staining.** Frozen sections were stained with biotinylated SNA followed by CY3-streptavidin and viewed under epifluorescence. The low level of reactivity seen in some tissues of the ST6Gal-I null mice was not higher than the background staining seen with the secondary reagent alone. *Bar* = 50 μm.

Altered Terminal Glycosylation in ST3Gal-I Null Mice—A survey of embryonic and young adult tissues of ST3Gal-I null mice with hematoxylin and eosin staining showed no gross histologic differences (data not shown). There were also no consistent differences seen in staining with MAL (data not shown). However, there were very clear differences observed with PNA and MAH (see below). The ST3Gal-I null animals could lack expression of some terminal α 2–3-linked sialic acids, thereby allowing PNA to bind the exposed Galβ1–3GalNAc termini. Conversely, binding of MAH, which recognizes α2–3-linked sialic acid, could be absent or decreased in such cell types. Indeed, there was a marked increase in intensity of binding to the PNA lectin in many organs of both day 16.5 embryos (Fig. 2) and the adult animals (see Figs. 4 and 6 for examples and Tables II and III for a summary). There was particularly intensified staining in specific areas within certain organs such as the thymus, kidney, and adrenal. Although the PNA-stained thymus of wild-type mice showed a clear border delineating the positive cortex and the negative medulla, the entire organ was stained in the null animals (Fig. 6). In the kidney, there was increased binding of the PNA lectin specifically to the kidney glomeruli of the ST3Gal-I null adults (Fig. 6) and also in the day 16.5 embryos (not shown). PNA also specifically highlighted the glomeruli in the kidneys of the ST3Gal-I null adults (Fig. 6) and day 16.5 embryos (seen under higher magnification, not shown). Interestingly PNA selectively and intensely stained only the medulla but not the cortex of the adrenal gland in wild-type mice, a situation converse to that seen in the normal thymus. In ST3Gal-I null animals there was a loss of distinction between the adrenal cortex and the medulla, as both were intensely stained with PNA (Fig. 6). Noticeably, the adrenal medulla of the ST3Gal-I null animals showed an almost complete loss of staining with MAH, indicating that ST3Gal-I may be the sole enzyme responsible for capping Galβ1–3GalNac residues throughout this endocrine organ (Fig. 6). Future studies of the aged ST3Gal-I animals may indicate whether this loss contributes to any endocrine physiological abnormalities. MAH also showed a moderate decrease in staining intensity of T cell areas in the spleen (Fig. 4), lymph nodes, and thymic medulla of the ST3Gal-I null animals (See table in Supplemental Material; no corresponding figure shown). None of the other organs showed significant changes in MAH binding (data not shown). These results were duplicated using more sensitive fluorescent secondary reagents (data not shown). No differences in staining with either SNA or ECA were observed between the wild-type and ST3Gal-I null organs (Fig. 4 and data not shown). As shown in Fig. 5, there was increased staining in the spleen of the ST3Gal-I null mice with tomato lectin. This lectin recognizes linear polylactosamine units, which are presumably extended on *O*-glycans in the absence of capping by ST3Gal-I.

Taken together, these data show that although the loss of

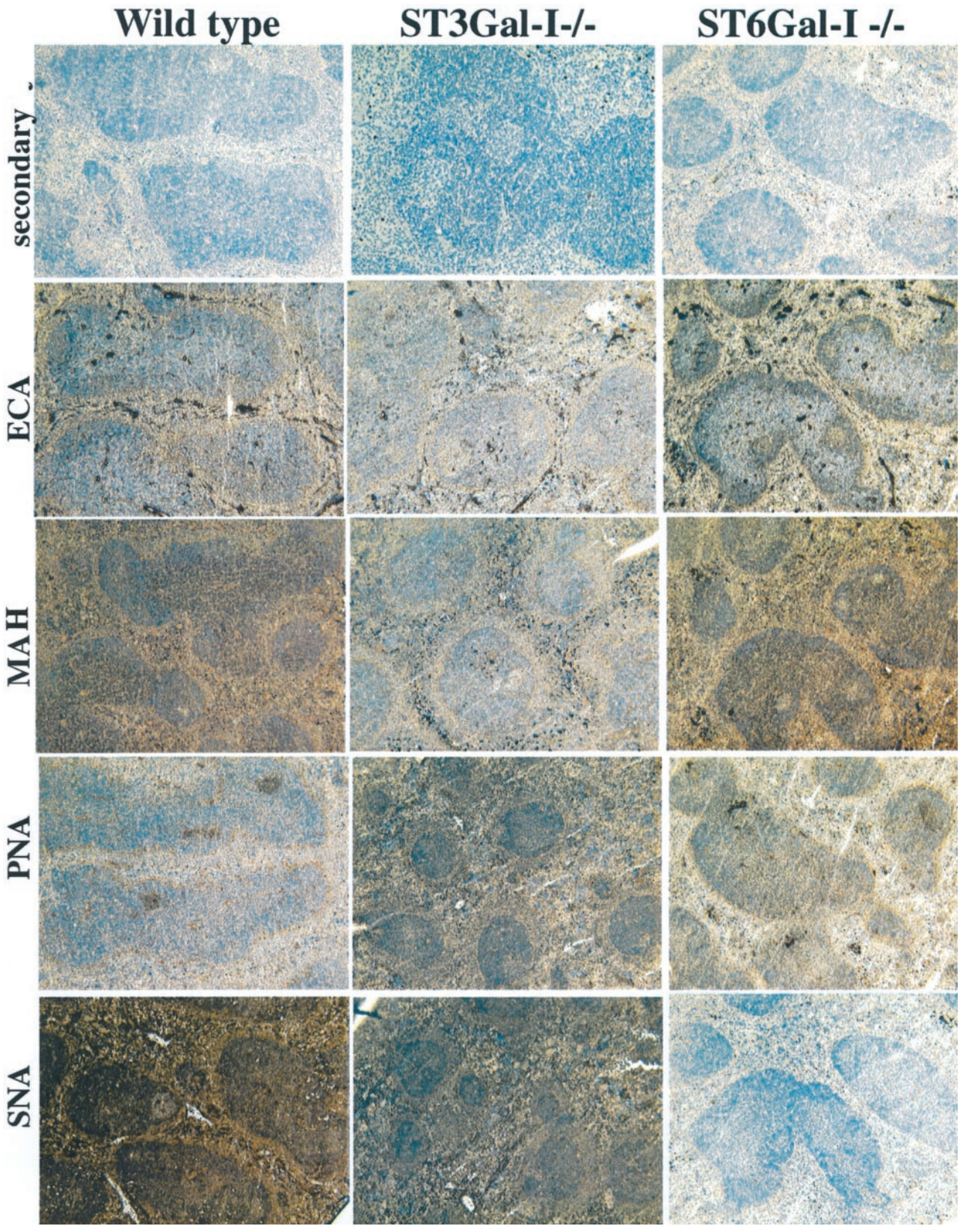

FIG. 4. **Changes seen in adult spleens of ST3Gal-I and ST6Gal-I null animals using lectin histochemistry.** Serial frozen sections of spleens from wild-type, ST3Gal-I, and ST6Gal-I null animals were stained with various biotinylated lectins followed by HRP-labeled streptavidin, color development, and hematoxylin counter-staining. The *panels* labeled "*secondary*" were exposed to HRP-labeled streptavidin only. *Bar* = 50 μm.

ST3Gal-I results in many exposed Galβ1–3GalNAc residues, there are other α2–3 STs active in most tissues. The other possibility is that the galactose residues are capped with 3-*O*-sulfate esters, which can also generate MAH binding (45). The striking exception is the adrenal gland, where almost all of the MAH binding is eliminated, suggesting that Siaα2–3Galβ1–3GalNAc synthesized by ST3Gal-I is the major sialylated epitope in this tissue.

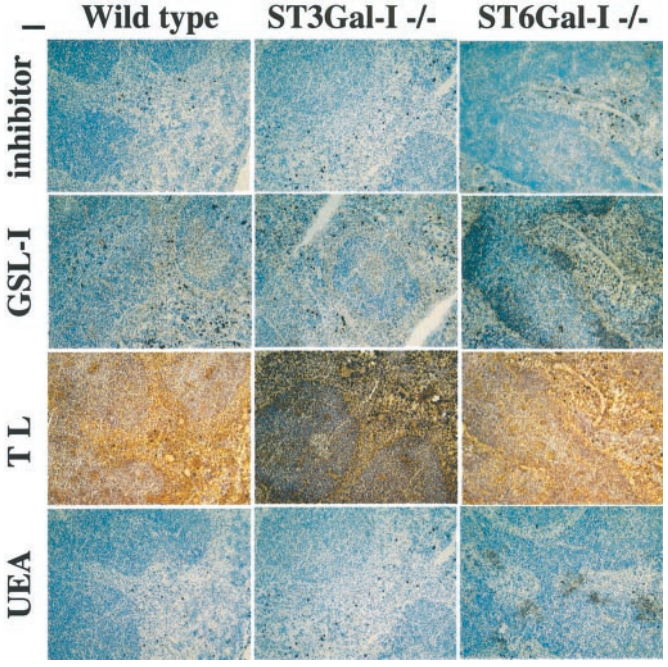

FIG. 5. **Changes seen in frozen sections of adult spleen using GSL-1, tomato lectin, and UEA histochemistry.** Serial sections of spleens from wild-type, ST3Gal-I, and ST6Gal-I null animals were stained with various biotinylated lectins followed by HRP-labeled streptavidin, color development, and hematoxylin counter-staining. The *panels* labeled "*inhibitor*" were exposed to lectins in the presence of inhibitor and then HRP-labeled streptavidin only. *Bar* = 50 microns.

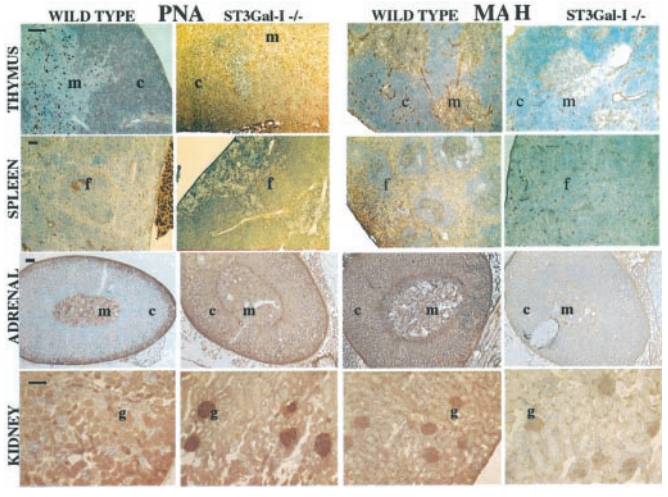

FIG. 6. **Changes seen in specific organs of ST3Gal-I null mice using lectin histochemistry.** Binding of biotinylated PNA or MAH to frozen sections of various organs from wild-type and ST3Gal-I null mice was detected with HRP-streptavidin, color development, and hematoxylin counter-staining. *m*, medulla; *c*, cortex; *f*, follicle; *g*, glomeruli. HRP-streptavidin alone gave no staining (not shown). *Bar* = 50 μm.

Altered Sialic Acid 9-O-Acetylation in ST6Gal-I and ST3Gal-I Null Mice—We previously presented evidence that sialic acid *O*-acetylation in cultured cells is regulated in a sialic acid linkage-specific fashion, apparently by the action of multiple sialic acid-specific *O*-acetyltransferases (46, 47). Wild-type murine CD4 positive cells derived from lymph nodes were also found to be preferentially 9-*O*-acetylated (26) compared with the CD8 positive population (demonstrated in Fig. 7, 90 *versus* 14%). ST6Gal-I null mice showed a similar pattern. Thus, despite loss of the Siaα2–6Galβ1–4GlcNAc sequence on their surfaces (35), $CD4^+$ lymphocytes of the ST6Gal-I null mice displayed nearly wild-type levels (82%) of 9-*O*-acetylation

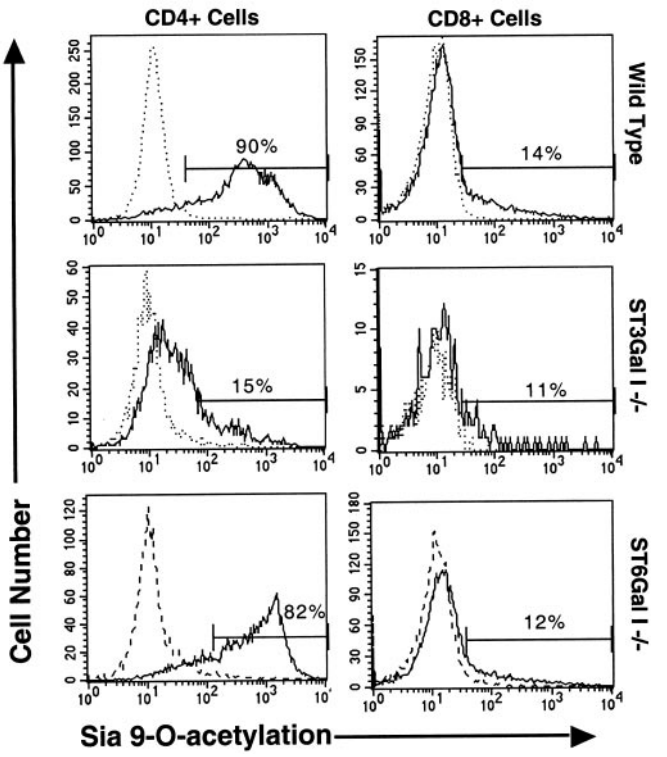

FIG. 7. **Flow cytometry analysis of T cells for detection of 9-*O*-acetyl sialic acids (*Sia*).** Lymph node-derived T-cell subsets from 4–6-week-old ST6Gal-I null and ST3Gal-I null mice and their wild-type littermates were analyzed for expression of surface 9-*O*-acetylated sialic acids using anti-CD4, anti-CD8 and CHE-FcD. Wild-type and ST6Gal-I null CD4 positive cells were preferentially 9-*O*-acetylated compared with CD8 positive cells (n = 6). In contrast, CD4 cells of the ST3Gal-I null mice showed a marked decrease in surface 9-*O*-acetylated sialic acids (n = 7). Similar results were obtained using thymic, splenic, or peripheral blood-derived lymphocytes (data not shown). Fluorescence signal intensity using phycoerythrin-conjugated FcD is shown for CD4-gated (*left panels*) and CD8-gated (*right panels*) lymphocytes, and the percentage of positive cells is indicated. *Dotted lines* reflect signal intensity using the esterase (nonbinding) form of the influenza C hemagglutinin esterase probe conjugated to phycoerythrin as a negative control.

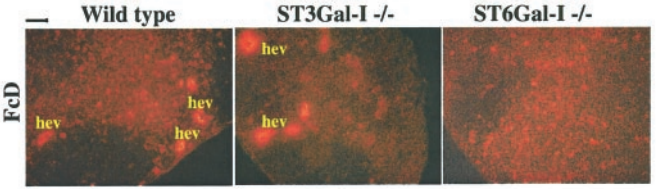

FIG. 8. **Detection of 9-*O*-acetylation of sialic acids in lymph node sections.** The CHE-FcD probe was pre-complexed with phycoerythrin-conjugated goat anti-human IgG at an optimized ratio and applied to frozen, acetone-fixed, blocked sections of lymph nodes from wild-type, ST3Gal-I, or ST6Gal-I null animals for 1 h at 4 °C. Sections were then washed and mounted with Aquamount for viewing. Examples of high endothelial venules are marked *hev*. The punctate staining seen represents lymphocytes within the parenchyma of the nodes. *Bar* = 50 μm.

compared with CD8 cells (12%). In contrast, the ST3Gal-I null mouse showed a dramatic decrease in 9-*O*-acetylation in the $CD4^+$ population (15%) compared with both wild-type and ST6Gal-I null mice (Fig. 8). The low levels of 9-*O*-acetylation seen in the wild-type and ST6Gal-I null CD8 population is further diminished in the ST3Gal-I null animals. We found similar patterns of change in 9-*O*-acetylation on T cells derived from thymus, spleen, and peripheral blood of ST3Gal-I null mice (data not shown). These data confirm the previous suggestion that 9-*O*-acetylated sialic acids on murine lymphocytes

are carried primarily on mucin-type glycoproteins with *O*-linked glycans (26). Thus, the loss of the ST3Gal-I enzyme has evidently eliminated a target selective for a specific 9-*O*-acetyltransferase in this particular cell type.

Tissue sections stained with the CHE-FcD probe for 9-*O*-acetylated sialic acids showed corresponding selective changes in the two types of null animals. In the wild-type mouse, CHE-FcD stained T-lymphocyte-rich areas in the spleen, thymus, and lymph nodes. It also bound well to endothelial cells in most organs examined and to certain areas of the brain, adrenal medulla, and pancreas (summarized in Table III in the Supplemental Material, detailed data not shown). The lymph nodes of the ST6Gal-I null mice showed wild-type levels of staining of T-cell zone lymphocytes (Fig. 8). However, there was a specific loss of endothelial cell (high endothelial venule) staining by the CHE-FcD probe. This selective loss of 9-*O*-acetylation in vascular endothelium was also true in all other tissues studied (data not shown). Thus, the expression of the ST6Gal-I product is required for the expression of 9-*O*-acetylation only in endothelial cells. Conversely, in the ST3Gal-I null mice, there was a decrease in intensity of CHE-FcD staining of T-cell zone lymphocytes in lymph nodes but no decrease in endothelial staining. Fig. 8 shows an example of these findings in lymph nodes, where the staining of the T-cell-rich zone was selectively lost in the ST3Gal-I null animals and the staining of the high endothelial venules was lost in the ST6Gal-I null animals.

Conclusions and Perspectives—We hypothesized that lectin histological studies of mice with genetically altered STs would reveal important clues regarding the enzymatic regulation of terminal glycosylation in the Golgi apparatus of an intact mammalian organism. These analyses have indeed provided insight into the structural features of glycans in defined genetic and mutant backgrounds. Despite the apparent normality of the tissues upon routine histological analyses, there are multiple changes in glycan structure uncovered by the lectin analysis. Taken together, our data indicate that *in vivo* regulation of sialic acid linkages and sialic acid 9-*O*-acetylation is indeed affected by the action of ST6Gal-I and ST3Gal-I but only in a cell type-specific manner. Likewise, the level of competition for newly available acceptor sites varies according to the specific ST that is missing and the cell type in question.

Most glycosyltransferases studied to date are members of multigene families with overlapping specificities. However, the complete loss of binding of SNA in almost all cell types and tissues of the adult ST6Gal-I null mouse indicates that this is the only enzyme in the mouse that can produce the Siaα2–6Galβ1–4GlcNAc linkage. Nevertheless, the absence of this enzyme does not result in a large excess of exposed Galβ1–4GlcNAc termini in the adult animal. Instead, there was an increase in the amount of terminal Galα1–3Galβ1–4GlcNAc and Fucα1–2Galβ1–4GlcNAc units. Another explanation is that ST6Gal-I selectively sialylates only certain termini on *N*-glycans (48). Thus, the loss of ST6Gal-I is not necessarily expected to result in a marked increase in exposed termini. With regard to there being no obvious increase in MAH staining, we emphasize that whereas the appearance of or marked loss of lectin staining is easy to define, modest increases are not. In other words, when significant staining is already present at base line, it is difficult to quantitate moderate increases in staining.

SNA can also recognize the Siaα2–6GalNAcα1-*O*-Ser/Thr (Sialyl-Tn) structure that is generated by the ST6GalNAc-I and -II enzymes (49, 50). This is true whether or not the sialic acid is in the *N*-glycolyl form and/or is 9-*O*-acetylated (43). High expression of this antigen is associated with a poor prognosis in most cancers studied (51–55). The almost complete absence of SNA staining in the young adult ST6Gal-I null mouse allows us to say that the expression of the sialyl-Tn epitope is very rare and may indeed be relatively specific for the malignant state. The traces of SNA positivity in the ST6Gal-I null embryo may represent the fetal expression of this antigen.

In contrast to the situation with ST6Gal-I, ST3Gal-I is one member of a family of ST3Gal isozymes, some of which are known to be able to compete with each other (20, 21, 23, 24). MAH staining could also be retained following elimination of ST3Gal-I if the terminal Gal residues were now subjected to 3-*O*-sulfation (45). Thus, it is not too surprising that MAH staining was substantially retained in the absence of ST3Gal-I (with a few dramatic exceptions, such as in the adrenal gland). However, in this case, compensation by other capping enzymes is quite incomplete, giving rise to markedly increased PNA staining in many tissues.

The differential expression of the mRNA for α2–3- and α2–6-sialyltransferases has been described in rat and human tissues (21, 56) and does not correlate in all instances. Furthermore, expression of mRNA does not always correlate with actual enzyme levels, which in turn, cannot always predict glycosylation. This study, the first to describe comprehensively cell type-specific expression of different linkages of terminal sialic acids in a variety of mouse tissues, forms a foundation for the histologic interpretations of future examples of sialyltransferase knock-out mice.

Finally, our findings also reveal that certain STs can regulate the expression of sialic acid 9-*O*-acetylation in a highly tissue-specific fashion. Thus, expression of ST6Gal-I provides selective targets for 9-*O*-acetylation in endothelial cells throughout the body, whereas the ST3Gal-I enzyme seems to provide targets only in $CD4^+$ lymphocytes. The precise role of 9-*O*-acetylation on endothelium or on $CD4^+$ T cells has not been defined. However, in other instances 9-*O*-acetylation has been known to block recognition by Siglecs or by Factor H of the complement pathway and to either block or facilitate the recognition of sialic acids by viral hemagglutinins (16).

Further studies are needed to clarify the genetic and cell biological bases of many of these regulatory events. Meanwhile, these studies have pointed to cell types and tissues in which there could be additional biological consequences of these ST deficiencies beyond those seen on B- and T-lymphocytes, *e.g.* the adrenal gland and kidney in ST3Gal-I null mice and several tissue types in ST6Gal-I null mice.

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