Natural Ligands of the B Cell Adhesion Molecule CD22β can be Masked by 9-*O*-Acetylation of Sialic Acids

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Abstract. CD22β is a B cell–restricted phosphoprotein expressed on the surface of mature resting B cells. It mediates interactions with other cells partly or exclusively via recognition of α2-6–linked sialic acids on glycoconjugates. The sialylated N-linked oligosaccharides recognized best by CD22β are common to many glycoproteins, suggesting that additional regulatory mechanisms may exist. Since the exocyclic side chain of sialic acid is required for recognition, we explored the effects of a naturally occurring modification of the side chain, 9-*O*-acetylation. Semisynthetic N-linked oligosaccharides terminating with 9-*O*-acetylated, α2-6–linked sialic acids showed markedly reduced binding to CD22β relative to their non–*O*-acetylated counterparts. Murine lymphoid cells were probed for natural CD22β ligands that might be *O*-acetylated using recombinant soluble forms of CD22β (CD22βRg) and influenza C esterase (CHE-Fc, which specifically removes 9-*O*-acetyl esters from sialic acids). By flow cytometry analysis, CD22βRg binding to splenic B cells and a subset of T cells was increased by pretreatment with CHE-Fc, indicating that some potential CD22β ligands are naturally "masked" by 9-*O*-acetylation. Unmasking of these CD22β ligands by removal of 9-*O*-acetyl esters from intact splenocytes substantially increases their CD22β-dependent adhesion in an in vitro adhesion assay. Probing of murine lymphoid tissue sections by CD22βRg and CHE-Fc treatment demonstrates regionally restricted and differentially expressed patterns of distribution between masked and unmasked ligands. For example, lymph node–associated follicular B cells express high levels of CD22β ligands, none of which are masked by 9-*O*-acetylation. In contrast, the ligands on lymph node-associated dendritic cells are almost completely masked by 9-*O*-acetylation, suggesting that masking may regulate interactions between CD22β-positive B cells and dendritic cells. In the thymus, only medullary cells express CD22β ligands, and a significant portion of these are masked by 9-*O*-acetylation, particularly at the cortical–medullary junction. Thus, 9-*O*-acetylation of sialic acids on immune cells is in a position to negatively regulate CD22β adhesion events in a manner depending on both cell type and tissue localization.

INTERCELLULAR adhesion between leukocytes involves a variety of receptor/ligand pairs that are required for eliciting immune responses (37). One such adhesion molecule is a B cell–restricted glycoprotein of the immunoglobulin superfamily, CD22β (7, 8, 38, 39, 49). First expressed within the interior of pre–B cells, CD22β is mobilized to the cell surface during maturation, coinciding temporally with the surface expression of IgD (7). Two human CD22 cDNAs designated CD22α and CD22β have been isolated, and their predicted sequences share homology to several adhesion molecules, including the neural cell adhesion molecule, myelin-associated glycoprotein, and carcinoembryonic antigen (38, 49). The two differ only in that CD22β contains seven extracellular Ig domains, whereas CD22α lacks Ig domains 3 and 4 (38, 49). While some differences in the binding properties of the two recombinant forms have been reported, CD22β appears to be the predominant cell surface form in both human and murine systems (41). Much evidence indicates the involvement of CD22β in B cell proliferation. A fraction of CD22β coexists with surface IgM on the surface of naive B cells (7). In response to IgM cross-linking, CD22-positive lymphocytes show selective increases in calcium flux (26), specific cytoplasmic tyrosine residues on CD22β are rapidly phosphorylated (32), and the cell surface expression of CD22β is quickly enhanced beyond basal levels. However, over longer time periods, surface expression is strikingly reduced (7, 20, 32). Cross-linking of CD22β facilitates the calcium mobilization induced by cross linking surface IgM (25).

On T cells, various isoforms of the common leukocyte an-

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tigen CD45 are ligands for CD22β (2, 39). A recombinant soluble form of CD22β precipitates CD45, as well as a number of other lymphocyte glycoprotein ligands (34). Although adhesion molecules with extracellular Ig domains generally mediate recognition by protein–protein interactions, the common feature of all CD22β-mediated interactions is an absolute requirement for sialic acids as a part of the ligand (2, 8, 27, 28, 34, 39). In this regard, we have demonstrated that CD22β specifically interacts with free oligosaccharides (derived from either the natural ligands or prepared synthetically) that terminate with one or more α2-6–linked sialic acids, and that other anomeric linkages do not confer interaction (27, 28). Since the expression of the α2-6 sialyltransferase (α2-6 STN,[1] which selectively attaches sialic acid in α2-6 linkage to N-linked oligosaccharides) is relatively widespread, such α2-6–sialylated-oligosaccharides are relatively common on glycoproteins. This raises the question of how CD22β selectively recognizes its specific counterreceptors. One possibility is that α2-6–linked sialic acids are sometimes modified in a manner that abolished interaction with CD22β. The term "sialic acid" actually refers to a diverse family of molecules derived from the nine-carbon acidic sugar *N*-acetyl-neuraminic acid (Neu5Ac, Fig. 1, structure *A*). Among the more common of these are modifications of the exocyclic side chain by *O*-acetyl ester groups at C7 and C9, generating 7-*O*-acetyl-*N*-acetyl neuraminic acid (Neu5, $7Ac_2$) and 9-*O*-acetyl-*N*-acetyl neuraminic acid (Neu5, $9Ac_2$), respectively (31, 44). Since *O*-acetyl esters at the C7 position spontaneously migrate to the C9 position at physiologic extracellular pH (16, 44), Neu5, $9Ac_2$ (Fig. 1, structure *D*) predominates on cell surface glycoconjugates (5). We have previously demonstrated that selective chemical oxidation of C8 and C9 (Fig. 1, structures *B* and *C*) by mild periodate treatment (27, 34) abolishes CD22β recognition. Thus, the natural side chain modification of 9-*O*-acetylation is a candidate for negative regulation of CD22β interactions.

To pursue this possibility, we used both conventional techniques, as well as a novel reagent derived from the influenza C virus 9-*O*-acetyl-sialic acid–specific esterase (17). We demonstrate here that 9-*O*-acetylation can indeed "mask" recognition of synthetic and natural ligands by CD22β, and that it can also abrogate CD22β-dependent cell adhesion. Additionally, immunohistological studies of lymphoid tissues show that potential ligands "masked" by 9-*O*-acetylation are present on specific cell types and in distinctive regional distributions.

Materials and Methods

Materials

Most of the chemicals and some of the reagents used were from Sigma Chemical Co. (St. Louis, MO). The following were obtained from the commercial sources indicated: diisopropyl fluorophosphate, Aldrich Chemical Co. (Milwaukee, WI); HPLC solvents, Fisher Scientific (Tustin, CA); asialo fetuin triantennary oligosaccharide (FT-02), Dionex Corporation; FITC-goat anti-human IgG_1, Cappel Laboratories (Cochranville, PA); phycoerythrin-conjugated antimurine Thy 1.2 (pan T cell marker) and phycoerythrin conjugated antimurine B220 (pan B cell marker), PharMingen (San Diego, CA). α2-6 STN was a generous gift from J. C. Paulson (Cytel, La Jolla, CA).

Synthesis of (CMP)-[^{3}H]Neu5Ac Derivatives

Cytidine monophosphate (CMP)-sialic acid synthase was purified from rat liver as described (29), and an enriched preparation from the 60% ammonium sulfate cut was used. [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ was synthesized by labeling isolated rat liver Golgi vesicles with [^{3}H-*acetyl*]acetylCoA, releasing sialic acids with *Arthrobacter ureafasciens* sialidase, and purifying them by ion exchange chromatography as described (45). [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ was purified away from unlabeled endogenous Neu5Ac by descending paper chromatography using ethanol/1 M ammonium acetate pH 6.5 (7:3) as a solvent system. Regions corresponding to [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ were eluted from paper in water and dried. To synthesize CMP-[9-^{3}H-*acetyl*]Neu5,$9Ac_2$, 2 μCi of [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ was dried and resuspended in 100 μl of 180 mM Tris, pH 7.0, 10 mM manganese chloride, 3 mM cytidine triphosphate, and 5 mM dithiothreitol (12). After addition of 100 μl of the CMP-sialic acid synthase preparation and incubation for 1 h at 37°C, 1 vol of ice-cold acetone was added to quench the reaction. Precipitates were removed by centrifugation at 13,000 *g* for 5 min, the supernatant was dried, redissolved in ethanol, and spotted onto paper alongside standard [9-^{3}H-*acetyl*]Neu5, $9Ac_2$ and CMP-[^{14}C]Neu5Ac. The paper chromatogram was developed overnight using the above solvent system, dried, cut into 1-cm strips (46 strips), and each was soaked in 1 ml of water. Aliquots (1%) of each were counted and fractions corresponding to CMP-[9-^{3}H-*acetyl*]Neu5,$9Ac_2$ (which migrates between CMP-[^{14}C]Neu5Ac and [9-^{3}H-*acetyl*]Neu5,$9Ac_2$) were pooled and dried. After purification, the final yield of CMP-[9-^{3}H-*acetyl*]Neu5,$9Ac_2$ relative to starting [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ was typically ~15%. Since the CMP-[9-^{3}H-*acetyl*]Neu5, $9Ac_2$ is relatively unstable, it was used within 1 wk of synthesis.

Synthesis of α2-6–linked Neu5,$9Ac_2$ on a Triantennary Oligosaccharide

Asialotriantennary oligosaccharide (1.5 μg) was dried along with 300,000 cpm of purified CMP-[9-^{3}H-*acetyl*]Neu5,$9Ac_2$, and was resuspended in 20 μl of 100 mM sodium cacodylate pH 6.9 with 1 mg/ml BSA. 1–2 mU of α2-6 STN was added and the reaction was allowed to proceed overnight at 37°C. This mixture was passed over an anion exchange column (Mono-Q; Pharmacia) to fractionate oligosaccharides by charge (27), with the pH of the elution buffer lowered to pH 7.0 to retain 9-*O*-acetyl esters. The [^{3}H-*acetyl*]-labeled sialylated oligosaccharides elute from this column in order of increasing numbers of sialic acid residues, and residual [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ and CMP-[9-^{3}H-*acetyl*]Neu5,$9Ac_2$ are resolved from these structures. In this manner, the number of [9-^{3}H-*acetyl*]Neu5,$9Ac_2$ groups incorporated into each oligosaccharide peak is known. After desalting each peak over a Bio-Gel P-4 column (BioRad Laboratories, Richmond, CA), these oligosaccharides were either directly passed onto the CD22βRg-PAS (chimeric protein made of the first three extracellular domains of CD22β, fused to the Fc portion of human IgG_1[CD22βRg] attached to protein A–Sepharose) column as described (27) or the remaining terminal β-galactose residues were sialylated to near completion by incubation with 0.1–0.2 mM nonlabeled CMP-Neu5Ac and 1–2 mU of α2-6 STN, giving triantennary oligosaccharides with two or three sialic acids in α2-6 linkage (assessed by Mono-Q chromatography). Thus, the number of [9-^{3}H-acetyl]Neu5,$9Ac_2$ and Neu5Ac groups incorporated into each labeled oligosaccharide is known. In certain experiments, complete sialylation with nonlabeled CMP-Neu5Ac was carried out without previous charge fractionation, resulting in a heterogenous mixture of oligosaccharides containing at least one and possibly two 9-*O*-acetyl groups. Each of these oligosaccharide preparations was assessed for interaction with a CD22βRg-PAS column as described (27). The column elution profiles were compared to those of corresponding non-*O*-acetylated sialylated derivatives passed over the same CD22βRg-PAS column run on the same day. In experiments following [^{3}H-acetyl] tracer exclusively incorporated into 9-*O*-acetyl esters, the results might be confusing if the labile esters became detached from the oligosaccharide. However, aliquots of radioactivity eluting from the CD22βRg-PAS column continued to void on a Bio-Gel P-6 column, demonstrating persistent attachment of the esters to the oligosaccharides (data not shown).

1. *Abbreviations used in this paper*: α2-6 STN, α2-6 sialyltransferase; CD22βRg, chimeric protein made of the first three extracellular domains of CD22β, fused to the Fc portion of human IgG_1; CD22βRg-PAS, CD22βRg attached to protein A–Sepharose; 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); CMP, cytidine monophosphate; Neu5Ac, *N*-acetyl-neuraminic acid; Neu5,$9Ac_2$, 9-*O*-acetyl-*N*-acetyl neuraminic acid; Neu5Gc, *N*-glycolyl-neuraminic acid; STN, sialyltransferase catalyzing attachment of Neu5Ac to N-linked oligosaccharides.

Synthesis of α2-6 sialylated Biantennary N-linked Oligosaccharides Terminated with [³H]Neu5Gc

CMP-[³H]*N*-glycolyl-neuraminic acid (Neu5Gc) was synthesized and purified as described above, except that the reaction was performed at pH 8.0, and magnesium chloride was substituted for manganese chloride (12). CMP-[³H]Neu5Gc was used as a donor to transfer [³H]Neu5Gc to the nonreducing termini of asialo-biantennary N-linked oligosaccharides using α2-6 STN as described above.

Isolation of Murine Spleen Cells

4-wk-old B6/SJLF$_1$ mice were killed by cervical dislocation, their spleens were scraped over a wire mesh into RPMI 1640 on ice, and the cell suspension was passed through gauze to remove debris. Cells were pelleted at 1,500 rpm at 4°C for 5 min in an IEC centrifuge, resuspended in 4 vol of 17 mM Tris, pH 7.2, 140 mM ammonium chloride for 3 min to lyse erythrocytes, repelleted, and resuspended in RPMI 1640 on ice. Cells isolated in this manner were 80–85% viable as judged by trypan blue dye exclusion.

Production of CD22βRg, CHE-Fc, and CHE-FcD

Recombinant human CD22βRg (39) was kindly provided by I. Stamenkovic (Massachusetts General Hospital, Boston, MA). 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 (17). The modified form CHE-FcD was generated by treating CHE-Fc with 1 mM diisopropyl fluorophosphate to inactivate the esterase as described (17, 22). CHE-Fc specifically releases 9-*O*-acetyl esters from sialic acids (i.e., it is an esterase), whereas CHE-FcD specifically recognizes and binds to 9-*O*-acetylated sialic acids (for more details see reference 17 and Fig. 1). Because of the human Fc tails, binding of CD22βRg or CHE-FcD to murine tissues or cells can be detected with appropriate secondary reagents.

Mild Periodate Treatment of Isolated Cells

Cells isolated as described were resuspended in either ice-cold PBS, pH 7.0, containing 3 mM sodium metaperiodate or PBS alone (control cells) for 20 min on ice. The cells were washed 3× in PBS, pH 7.0, and were then treated with or without CHE-Fc (see below) to remove 9-*O*-acetyl esters from cell surface sialic acids. The cells were then stained for flow cytometry as described below.

Removal of 9-O-Acetyl Esters from Cell Surface Sialic Acids

About 5 × 10^6 cells were resuspended in 50 μl Tris-buffered saline, pH 7.0, containing 0.02% sodium azide (control cells) or 50 μl Tris-buffered saline, pH 7.0, containing 0.02% sodium azide with 20 μg CHE-Fc. After incubation at 37°C on an end-over-end rotator for 45 min, the cells were washed 2× in ice cold PBS, and were then stained for flow cytometry as described below.

Flow Cytometry Analysis

Cells (2 × 10^6) having undergone sham treatment or treatment with mild periodate and/or CHE-Fc were washed in ice-cold PBS/0.02% sodium azide and incubated on ice in 100 μl of PBS/0.02% sodium azide/1% BSA containing 20 μg/ml of CD22βRg for 40 min. For double-staining analysis, phycoerythrin-conjugated rat antimurine B220 (IgG$_{2a}$) or rat-antimurine Thy1.2 (IgG$_{2a}$) were incubated along with CD22βRg at concentrations of 5 μg/ml. Cells were washed twice with ice-cold PBS/0.02% sodium azide, and were resuspended in 100 μl of PBS/0.02% sodium azide/1% BSA containing FITC-conjugated goat anti-human IgG (1:50 dilution of 1 mg/ml). After incubation for 40 min, cells were washed twice in PBS/0.02% sodium azide and fixed in PBS/0.02% sodium azide containing 2% paraformaldehyde. Isotype controls consisted of incubating cells with equivalent concentrations of human IgG$_1$ in place of CHE-Fc or CD22βRg. Stained fixed cells were analyzed on a FACscan® machine (Beckton Dickinson Immunocytometry Systems, Mountain View, CA).

Cell Binding to CD22βRg

Microtiter wells were coated overnight at 4°C with 0.1 mg/ml protein A in 50 mM Tris-Cl, pH 8.5, rinsed once with PBS, blocked for 3 h at 4°C with PBS, 1 mg/ml BSA, rinsed again, incubated with 50 μl/well of CD22βRg (0.1 mg/ml) in PBS for 2 h, and finally rinsed three times with PBS. Murine splenocytes, after treatment with or without mild periodate (as above) and/or CHE-Fc, were washed in PBS, and were added (50 μl at 10^7 cells/ml) to quadruplicate sets of wells. Cells were allowed to adhere for 30 min at 4°C, and nonadherent cells were removed by gently washing four times with ice-cold PBS. The center of each well where cells were uniformly attached was then photographed on an inverted microscope (×10 phase objective), and the number of cells in the resulting photomicrograph were counted. As controls for nonspecific binding, cells were treated (subsequent to initial CHE-Fc treatment) with mild periodate as above and then added to wells containing CD22βRg. The binding of non-periodate-treated cells to wells lacking CD22βRg (protein A alone) was also examined.

Immunohistological Analysis of Murine Lymphoid Tissues with CHE-FcD and CD22βRg

Tissues were obtained from 4-wk-old B6/SJLFl mice that were killed as described above, and were immediately suspended in OCT and frozen in liquid nitrogen. Freshly cut frozen sections from cervical lymph node, spleen, and thymus were fixed in ice-cold acetone (10 min), endogenous peroxidase quenched with 0.03% hydrogen peroxide in PBS (10 min), and then incubated at 4°C (20 min) with blocking solution (3% goat serum/1%BSA in HBSS). Sections were washed three times in HBSS and overlaid with 3 mM sodium metaperiodate in PBS or PBS alone (sham treatment) for 20 min at 4°C and washed three times again in HBSS. Sections were then treated with CHE-Fc (20 μg/ml) in blocking solution or blocking solution alone (controls) at room temperature for 45 min, washed three times again in HBSS and overlaid with CHE-FcD, CHE-Fc, or CD22βRg (20 μg/ml) in blocking solution at 4°C for 1 h, after which they were washed three times again in HBSS. Detection was achieved by sequential incubations with biotinylated goat anti-human IgG (1:100 dilution in blocking buffer) and streptavidin-peroxidase (1:100 of a 1 mg/ml solution in blocking buffer) for 30 min at room temperature with three HBSS washes after each incubation. Development was achieved with 3-amino-9-ethylcarbazole-peroxide and hematoxylin counterstain. In these analyses, mild periodate treatment is used as a negative control for CD22βRg staining (see Fig. 1 for rationale), and CHE-Fc is used as a negative control for CHE-FcD staining. Mild periodate treatment does not nonspecifically inhibit the colorimetric development because high endothelial venule staining with L-selectin receptor globulin done in parallel (data not shown) is actually enhanced by this treatment (23). Note that acetone fixation was used because formaldehyde fixation results in decreased binding of CD22βRg (unpublished observations); consequently, the preservation of cellular detail is somewhat less than optimal. However, the resolution is sufficient to permit the conclusions reached.

Results

9-O-Acetylation of α2-6-linked Sialic acid on N-linked Oligosaccharides Abolishes Binding to CD22βRg

Using a sensitive column assay with CD22βRg bound to protein A–Sepharose, we previously demonstrated that free N-linked oligosaccharides with α2-6-linked sialic acids at their nonreducing termini bind to CD22β. The apparent binding affinity increases with increasing numbers of α2-6-linked residues, and it is abolished by their selective oxidation with mild periodate (see Fig. 1 for chemistry of reaction) (27, 28). To determine if 9-*O*-acetylation of α2-6-linked sialic acids on CD22β ligands might also influence binding (see Fig. 1 for structure), we used CMP-[³H-*acetyl*]Neu5,9Ac$_2$ and α2-6 STN to sialylate an asialotriantennary oligosaccharide, creating α2-6-sialylated structures with varying numbers of [³H-*acetyl*]Neu5,9Ac$_2$ residues (12, 48). We chose to incorporate radiolabel into the *O*-acetyl moiety rather than the sialic acid core because these esters are known to be somewhat labile (44). Thus, only oligosaccharides containing *O*-acetyl groups would be detected and analyzed. After sialylation, the oligosaccharides were fractionated by charge into species containing one, two, or three [³H-*acetyl*]Neu5,

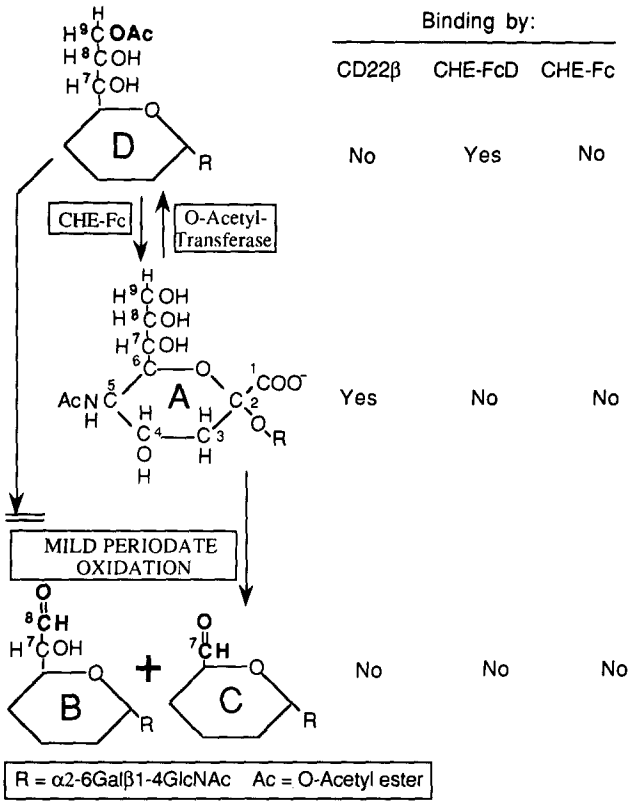

Figure 1. Natural and synthetic modifications of sialic acid and their interactions with recombinant derivatives of CD22β and influenza CHE. The most common form of sialic acid, Neu5Ac (structure *A*), may be naturally modified by an *O*-acetyl-transferase to produce Neu5,9Ac_2 (structure *D*). Under conditions of mild periodate oxidation (3 mM, pH 7.0, 4°C), the exocyclic side chain of Neu5Ac is selectively oxidized to produce eight and seven carbon products (structures *B* and *C*, respectively). The 9-*O*-acetyl group protects the side chain of Neu5,9Ac_2 from oxidation. The binding of oligosaccharides with α2-6 linked Neu5Ac residues to CD22β, as well as its abrogation by periodate oxidation, have been previously demonstrated (27, 28). The inability of oligosaccharides with α2-6-linked Neu5,9Ac_2 to bind to CD22β is demonstrated in Figs. 2–4. The binding of CHE-FcD to glycoconjugates containing Neu5,9Ac_2 residues has been demonstrated (17). CHE-Fc hydrolyzes the 9-*O*-acetyl groups (shown by the arrow from structure *D* to structure *A*), and it does not bind to any of these structures (17). For simplicity, only the relevant portions of structures *B*, *C*, and *D* are not shown.

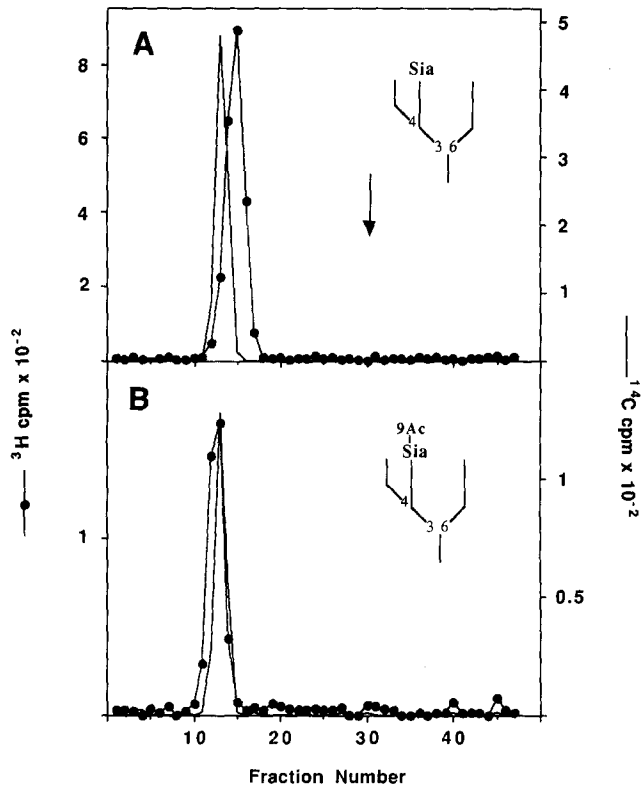

Figure 2. CD22β interactions with monosialylated triantennary oligosaccharides. Monosialylated triantennary oligosaccharides containing either α2-6–linked [^{3}H]Neu5Ac (*A*) or α2-6–linked [^{3}H-*acetyl*]Neu5,9Ac_2 (*B*) were prepared as described in Materials and Methods. Each sample was mixed with [^{14}C]ManNAc (as a marker for elution of nonbinding structures) and passed over a CD22βRg-PAS column at 4°C. The arrow represents the point at which the column was warmed and eluted further at ambient temperature. The inset structures represent the predicted sites of sialic acid addition by α2-6 STN, based on the branch specificity of this enzyme, as described in reference 14. *Sia*, Neu5Ac; *9Ac*, a 9-*O*-acetyl ester.

9Ac_2 residues. Portions of the molecules containing only one [^{3}H-*acetyl*]Neu5,9Ac_2 residue were then sialylated to completion with nonlabeled CMP-Neu5Ac (non-*O*-acetylated) and α2-6 STN.

The binding of these structurally defined oligosaccharides to CD22βRg was examined using the column assay. As demonstrated previously (27, 28), the elution of an oligosaccharide containing a single α2-6–linked [^{3}H]Neu5Ac residue is slightly but consistently retarded relative to the nonbinding sugar [^{14}C]ManNAc, indicating a weak but significant binding (Fig. 2 *A*). However, a similarly prepared oligosaccharide with a single α2-6–linked Neu5,9Ac_2 residue coelutes with [^{14}C]ManNAc (Fig. 2 *B*), indicating that the *O*-acetyl group abrogated detectable binding. An oligosaccharide with two α2-6–linked Neu5Ac residues elutes much later on this column, and its elution is enhanced by warming the column to ambient temperature (Fig. 3 *A*) (27, 28). In marked contrast, a population of [^{3}H-*acetyl*]Neu5,9Ac_2-labeled oligosaccharides with two negative charges elutes much earlier (Fig. 3 *B*). In fact 70% of this material coelutes with the [^{14}C]ManNAc marker, and the remaining 30% coelutes at a position corresponding to structures with a single α2-6 Neu5Ac residue (Fig. 2 *A*). These slightly retarded oligosaccharides probably represent structures containing one [^{3}H-*acetyl*]Neu5,9Ac_2 and one Neu5Ac residue.

Oligosaccharides with three α2-6–linked Neu5Ac residues bind better (elute even later) than those with two such residues (compare Figs. 3 *A* and 4 *A*, and see references 27, 28). In contrast, an oligosaccharide with a single α2-6[^{3}H-*acetyl*]Neu5,9Ac_2 residue and two non-*O*-acetylated α2-6Neu5Ac residues elutes much earlier (Fig. 4 *B*). In fact, it elutes slightly earlier than a non-*O*-acetylated bisialylated structure, appearing just before warming the column to ambient temperature (compare Figs. 3 *A* and 4 *B*). Explanations for this include the possibility that the Neu5,9Ac_2 group in-

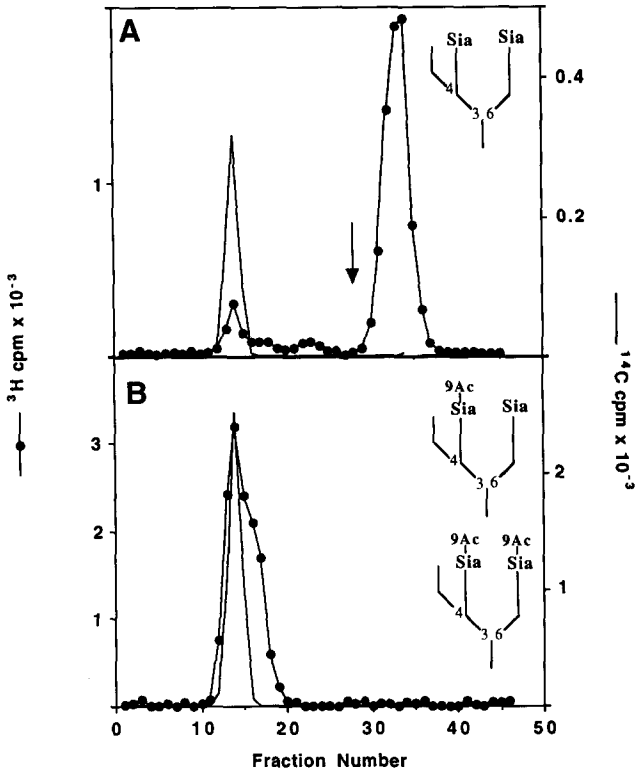

Figure 3. CD22β interactions with disialylated triantennary oligosaccharides. Disialylated oligosaccharide chains were prepared as described in Materials and Methods and passed over a CD22βRg-PAS column as in Fig. 2. Profiles are shown for (*A*) chains containing two [^{3}H]Neu5Ac residues and (*B*) a mixture of chains with one to two [^{3}H-*acetyl*]Neu5,9Ac$_2$ residue and zero to one Neu5Ac residues (see text for discussion). The inset structures represent the predicted sites of sialic acid addition for the major isomers by α2-6 STN. *Sia*, Neu5Ac; *9Ac*, a 9-*O*-acetyl ester.

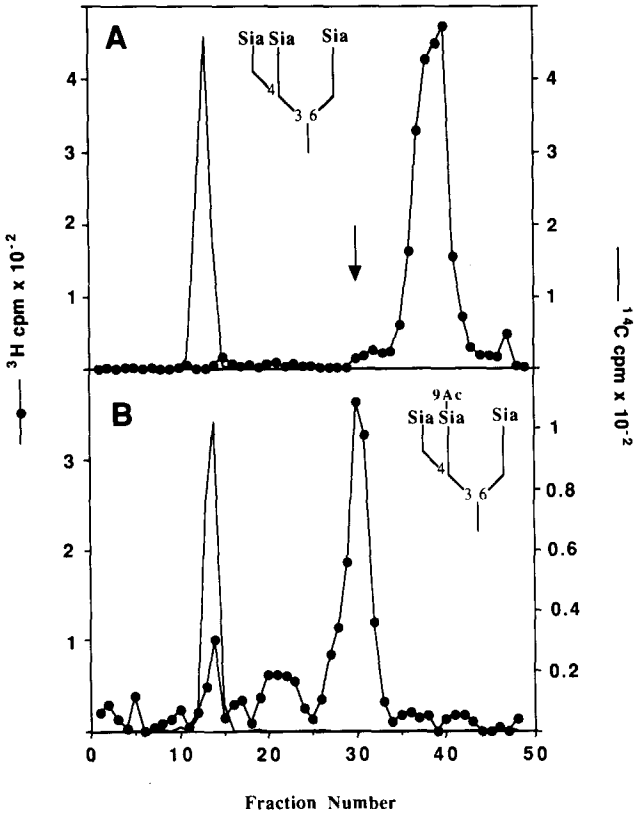

Figure 4. CD22β interactions between trisialylated triantennary oligosaccharides. Trisialylated triantennary oligosaccharides were prepared as described in Materials and Methods, and passed over a CD22βRg-Pas column as in Fig. 2. Profiles are shown for chains containing (*A*) three α2-6 linked [^{3}H]Neu5Ac residues or (*B*) one [^{3}H-*acetyl*]Neu5,9Ac$_2$ residue and two non-*O*-acetylated Neu5Ac residues. The inset structures represent the predicted sites of sialic acid addition by α2-6 STN. *Sia*, Neu5Ac; *9Ac*, a 9-*O*-acetyl ester.

terferes with the binding of the other two Neu5Ac residues, or that an oligosaccharide containing Neu5Ac residues on the outer antennae (as drawn in *inset*, Fig. 4 *B*), does not bind as tightly as those with two Neu5Ac residues on the two adjacent antennae (as drawn in the *insets*, in Fig. 3 *A*). Regardless, these observations clearly indicate that 9-*O*-acetylation of α2-6-linked sialic acids abolished their recognition by CD22βRg, and that the binding of multisialylated structures with a mixture of 9-*O*-acetylated and non-*O*-acetylated sialic acids is markedly reduced in comparison to similarly sialylated oligosaccharides with no 9-*O*-acetylated residues.

Another naturally occurring sialic acid modification is hydroxylation of the *N*-acetyl group that generates Neu5Gc- (31, 44). Since *N*-glycolyl-neuraminic acid is prevalent in mice, we asked if Neu5Gc in place of Neu5Ac alters interactions between CD22β and its ligands. Using CMP-[^{3}H]-Neu5Gc and α2-6 STN, biantennary oligosaccharides containing two α2-6-linked [^{3}H]Neu5Gc were constructed. Their binding to the CD22βRg-PAS column was indistinguishable from structures containing two Neu5Ac residues (data not shown). Thus, in contrast to 9-*O*-acetylation, this sialic acid modification has no inhibitory effect on CD22β recognition.

9-O-Acetylation Masks Potential Ligands for CD22β on Murine Splenocytes

Since expression of CD22 has been previously demonstrated in lymph nodes and splenic B cells (7, 41), these organs may also contain cells with ligands for this receptor. To search for such ligands and to examine the possibility that they might be masked by 9-*O*-acetyl groups, splenocytes were probed by flow cytometry for CD22βRg staining before and after treatment with the CHE-Fc esterase. If 9-*O*-acetylation of α2-6-linked sialic acids is masking potential CD22β ligands, CHE-Fc treatment should remove the esters (see Fig. 1), giving an increased fluorescence relative to non-treated cells. It is also known that 9-*O*-acetylation renders the sialic acid side chain resistant to mild periodate oxidation (see Fig. 1) (11). Thus, initial treatment of cells with mild periodate should eliminate CD22βRg staining, while potential ligands "masked" by 9-*O*-acetylation will remain intact. Subsequent treatment of cell surfaces with CHE-Fc should remove 9-*O*-acetyl esters, thereby unmasking these ligands (see Fig. 1). This sequential treatment protocol should accentuate the detection of "masked" ligands.

Two-color flow cytometry was performed with splenocytes stained with CD22βRg and either anti-Thy1.2 (for T

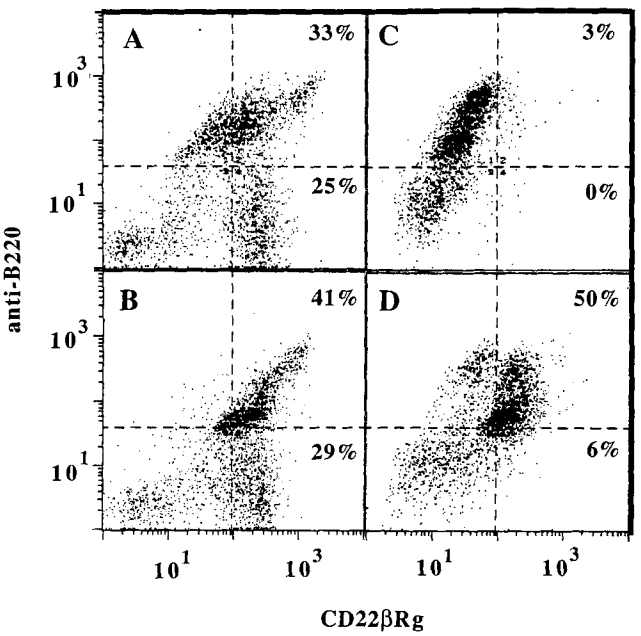

Figure 5. Double-staining flow cytometry analysis of murine splenocytes for CD22β ligands and Thy1.2. Murine splenocytes were isolated and either (*A*) sham treated; (*B*) treated with CHE-Fc; (*C*) treated with mild periodate, or (*D*) treated with mild periodate followed by CHE-Fc. After treatments, the splenocytes were stained with CD22βRg (FITC-conjugated secondary antibody) and phycoerythrin-conjugated anti-Thy1.2 for flow cytometry analysis as described in Materials and Methods. The number in the upper right hand corner of specified quadrants represents the percentage of cells from the entire population analyzed.

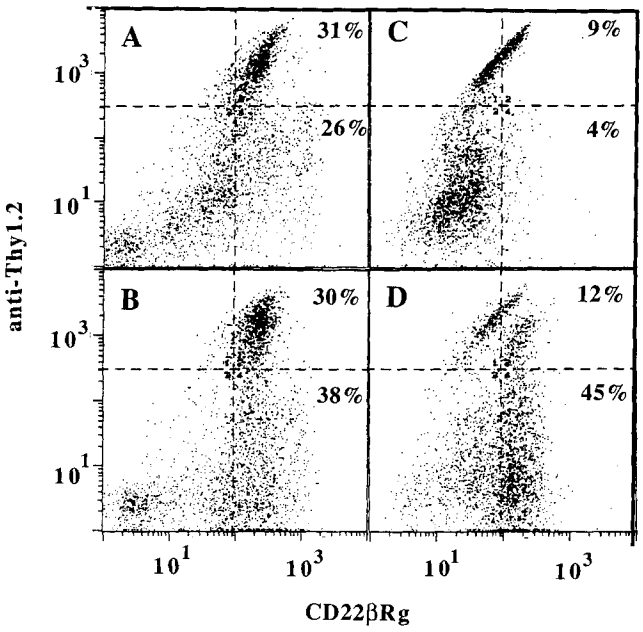

Figure 6. Double-staining flow cytometry analysis of murine splenocytes for CD22β ligands and B220. Murine splenocytes were isolated and either (*A*) sham treated, (*B*) treated with CHE-Fc, (*C*) treated with mild periodate, or (*D*) treated with mild periodate followed by CHE-Fc. After treatments, the splenocytes were stained with CD22βRg (FITC-conjugated secondary antibody) and phycoerythrin-conjugated anti-B220 for flow cytometry analysis as described in Materials and Methods. The number in the upper right hand corner of specified quadrants represents the percentage of cells from the entire population analyzed.

cells) or anti-B220 (for B cells). Almost the entire population of splenic T cells stained positive for CD22βRg ligands (Fig. 5 *A*). Upon CHE-Fc treatment, this staining does not obviously increase (Fig. 5 *B*). As expected, mild periodate treatment markedly reduced CD22βRg staining in all populations (although ~30% of Thy1.2-positive cells remained marginally CD22βRg positive; Fig. 5 *C*). Removal of 9-*O*-acetyl groups from the periodate-treated cells with CHE-Fc increased CD22βRg staining in Thy1.2-positive populations from 9–12% (Fig. 5 *D*), showing a small subpopulation with ligands masked by 9-*O*-acetylation. Thus, while the great majority of splenic T cells express high affinity ligands for CD22β, only a small subset of these express ligands masked by 9-*O*-acetylation. Mild periodate inactivation of native ligands before removal of 9-*O*-acetyl esters is required to clearly observe this population.

In contrast to the T cells, staining of Thy1.2-negative splenocytes (expected to be mainly B cells) markedly increased after removal of 9-*O*-acetyl esters (Fig. 5, *B* and *D*). In keeping with this, the majority of the B220-positive cells (B cells) stain significantly for CD22βRg ligands, and this population clearly increases upon treatment with CHE-Fc (Fig. 6, *A* and *B*). As illustrated by Fig. 6, *C* and *D*, previous treatment with mild periodate accentuates the demonstration of this effect of the esterase. This indicates that most but not all splenic B cells express potential ligands for CD22β. However, a significant portion of these ligands are masked by 9-*O*-acetylation.

Removal of 9-O-Acetyl Esters from Splenic Lymphocytes Increases CD22β-dependent Adhesion

As CD22βRg is believed to function as a cell adhesion molecule (39, 49), the effect of CHE-Fc esterase treatment on splenocyte binding to CD22βRg was examined in an adhesion assay. Isolated murine splenocytes, treated with CHE-Fc or buffer alone, were added to microtiter wells precoated with CD22βRg (immobilized with protein A to ensure correct orientation). Approximately one quarter of the added cells bound in the absence of esterase treatment (data not shown). CHE-Fc treatment resulted in a doubling of the number of adherent cells (Fig. 7). The binding of both CHE-Fc–treated and nontreated cells was specific for CD22βRg, as binding was reduced by >90% by either treating the cells with mild periodate, or by adding cells to wells lacking CD22βRg. Thus, the masking of ligands by 9-*O*-acetylation appears to be functionally significant in altering cell adhesion to CD22β.

In Situ Detection of Masked and Unmasked Ligands in Lymphoid Tissues

For initial clues to the functionality of this masking in immune processes, tissue sections from murine lymph node, spleen, and thymus were surveyed for CD22β ligands. The cellular phenotypes generating the morphological characteristics of lymphoid tissues are very well defined, particularly in the mouse (24, 46). We therefore identified cells express-

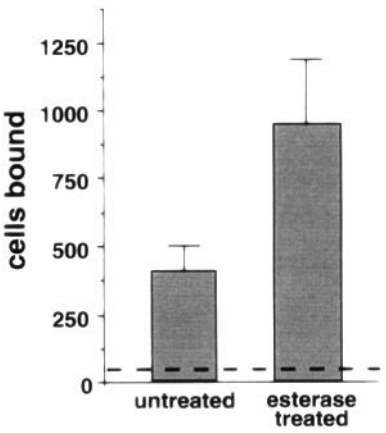

Figure 7. Effect of CHE-Fc esterase on CD22βRg-dependent adherence of splenocytes. Murine splenocytes were treated either with CHE-Fc esterase or with PBS alone, and then added (4×10^5 cells/well) to microtiter wells that had been precoated with CD22βRg. After 30 min at 4°C, the nonadherent cells were rinsed off with PBS, and the remaining adherent cells were quantitated as described in Materials and Methods. The binding of cells to wells lacking CD22βRg or the binding of mild periodate-treated cells to wells containing CD22βRg ranged from 2–9% of the level of binding between control cells and CD22βRg. The upper level of this nonspecific binding is indicated by the horizontal dashed line. Error bars indicate standard deviation ($n = 4$).

ing CD22β ligands on the basis of their anatomic locations, morphology, and hematoxylin-staining characteristics (lymphocyte nuclei stain darkly with hematoxylin counterstain, while dendritic/accessory cells stain relatively poorly and their morphology is distinctive). In parallel, we also examined the distribution of total 9-*O*–acetylated sialic acids (all 9-*O*-acetylated sialic acids, regardless of linkage) using the CHE-FcD probe (see Fig. 1 for rationale). As described elsewhere, CHE-FcD recognizes all 9-*O*–acetylated sialic acids, regardless of linkage or the type of glyconjugate to which they are attached (17). In this case, the enzymatically active CHE-Fc serves as a negative control because it cannot bind stably to 9-*O*–acetylated sialic acids at ambient temperatures (17).

CD22β Ligands Are Regionally Expressed on Marginal Zone Lymphocytes of Spleen and Are Enhanced by Removal of 9-O-Acetyl Esters

Total 9-*O*–acetylated sialic acids detected by CHE-FcD were found to be widely distributed in the spleen (Fig. 8 *A*). Within the marginal zone, a region enriched in B cells, only scattered lymphocytes reacted with this probe. However, lymphocytes in the periarteriolar lymphoid sheath, a region enriched in T lymphocytes, react strongly, indicating high levels of 9-*O*–acetylated sialic acids. Accessory cells surrounding vascular sinusoids in the red pulp and scattered throughout the periarteriolar sheaths also express 9-*O*–acetylated sialic acids. All this staining is specific for 9-*O*–acetylated sialic acids because sections are negative with CHE-Fc (Fig. 8 *B*). Although CHE-FcD does not detect substantial total 9-*O*-acetylation on marginal zone B cells, flow cytometry analysis (see above) and immunohistological staining with CD22βRg (see below) indicate that many splenic B cells do have CD22β ligands masked by 9-*O*-acetylation. Thus, these masking 9-*O*-acetyl groups must represent a small percentage of the total 9-*O*-acetylated sialic acids on splenic lymphocytes in general. In keeping with this, it has recently been shown that T cells express a variety of *O*-acetylated gangliosides, including 9-*O*-acetyl-G_{D3} (18, 19, 33). These would be detected by CHE-FcD, but not by CD22βRg.

In contrast to the widespread distribution of total 9-*O*–acetylated sialic acids in the spleen, CD22βRg ligands masked by 9-*O*-acetylation showed a regionally selective expression. Fig. 8 *C* shows that CD22βRg ligands are highly expressed on marginal zone B lymphocytes, but only on a minority of lymphocytes associated with the periarteriolar sheath (T cells, or B cells migrating to marginal zones). This pattern is strikingly enhanced by previous CHE-Fc treatment (Fig. 8 *E*), indicating that the cells in the same locations express masked ligands for CD22β. Accessory cells found throughout the red pulp also express ligands, some of which are masked by 9-*O*-acetylation (Fig. 8, *E* and *F*). Mild periodate pretreatment of sections abolished CD22βRg staining (Fig. 8 *D*), indicating the specificity of the results. When (9-*O*-acetyl groups were removed by CHE-Fc after mild periodate pretreatment, staining reappeared in the same regions as those that were enhanced by CHE-Fc treatment alone (data not shown). The enhancement by pretreatment with CHE-Fc is not caused by nonspecific CHE-Fc binding because incubations with CHE-Fc alone gave no significant staining (Fig. 8 *B*). These immunohistological analyses corroborate the results with flow cytometry and cell adhesion, which indicate that many splenic B cells express CD22β ligands masked by 9-*O*-acetylation. In addition, a few T cells, as well some accessory cells (which are not well represented in cells isolated for flow cytometry analysis), also express such masked CD22β ligands. The minor quantitative and qualitative differences between the results of flow cytometry, cell adhesion, and immunohistology most likely result from the differences in the sensitivity of the assays, and/or to selective recovery of cells.

CD22β Ligands in the Lymph Node are Regionally Expressed and Selectively Masked

Staining lymph node sections with CHE-FcD indicates that total 9-*O*-acetylated sialic acids are distributed mainly in paracortical regions, where T lymphocytes and interdigitating dendritic or accessory cells are typically found (24, 46) (Fig. 9 *A*). While some of this staining is caused by paracortical lymphocytes, the probe also stains irregularly shaped cells with indistinct nuclei in areas between lymphocytes, a pattern typical of interdigitating dendritic cells. Peripheral follicles, which are B cell–enriched regions, do not stain with CHE-FcD, except for a few cells with a dendritic cell morphology. Thus, in contrast to the spleen, follicular B cells in the lymph node do not seem to express 9-*O*–acetylated sialic acids. The marked staining seen in the lymph node hilum region is nonspecific (caused by adsorption of a reagent or an endogenous peroxidase not inactivated during processing) because it is also seen with the CHE-Fc negative control (Fig. 9 *B*). All other regions of the lymph node are devoid of staining with control CHE-Fc.

The expression of total 9-*O*-acetylated sialic acids in the

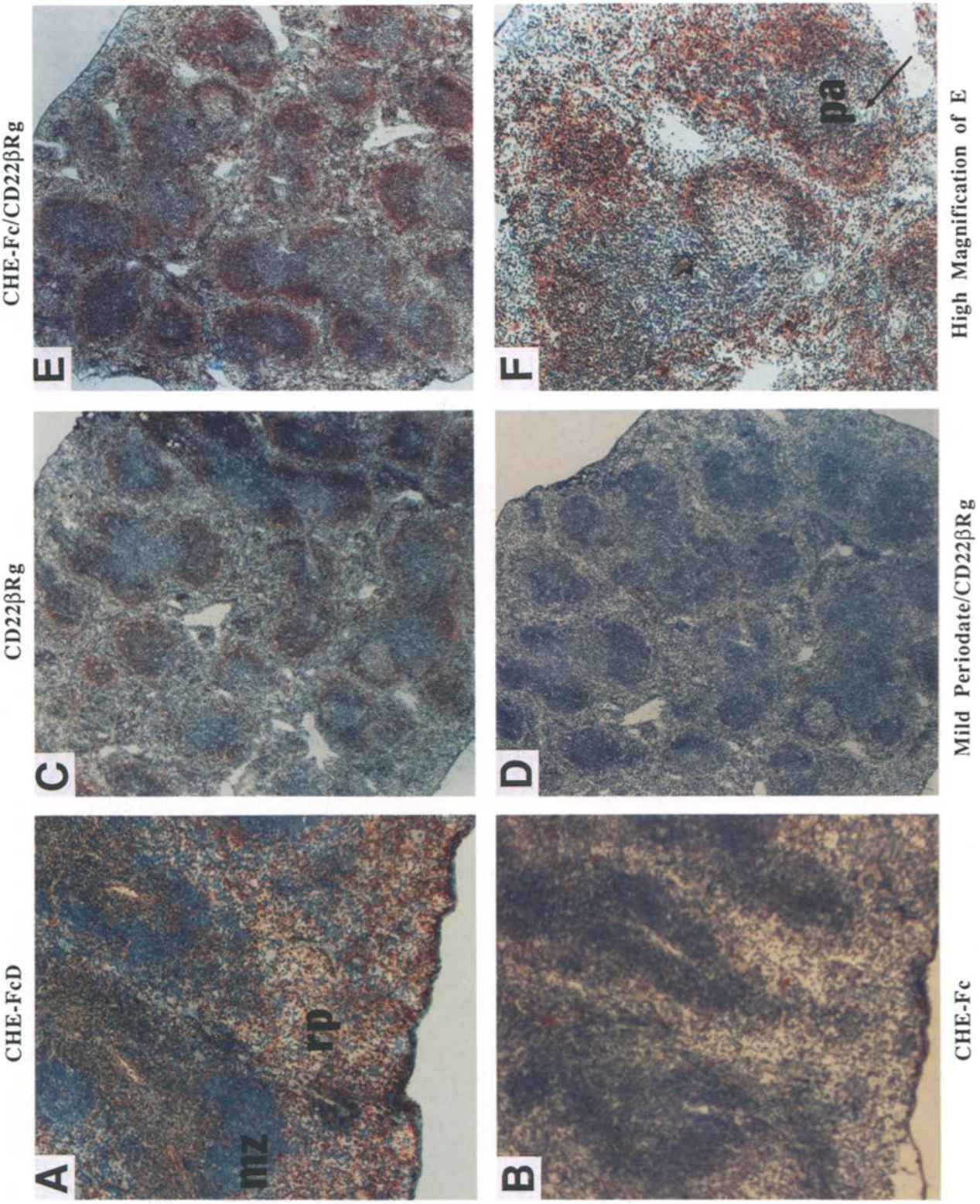

Figure 8. Immunohistological analysis of murine spleen. Frozen sections of murine spleen were fixed and probed with the specified reagents as described in Materials and Methods. (*A*) x125 CHE-FcD, (*B*) x125 CHE-Fc, negative control, (*C*) x50 CD22βRg, (*D*) x50 mild periodate treatment before CD22βRg staining, (*E*) x50 CHE-Fc treatment before CD22βRg, (*F*) x250 magnification of *E* showing the marginal zone. The arrow in *F* indicates a typical arteriole surrounded by a periarteriolar (pa) lymphoid sheath. Examples of the B cell–enriched marginal zone (mz) and the red pulp (rp) are indicated in *A*.

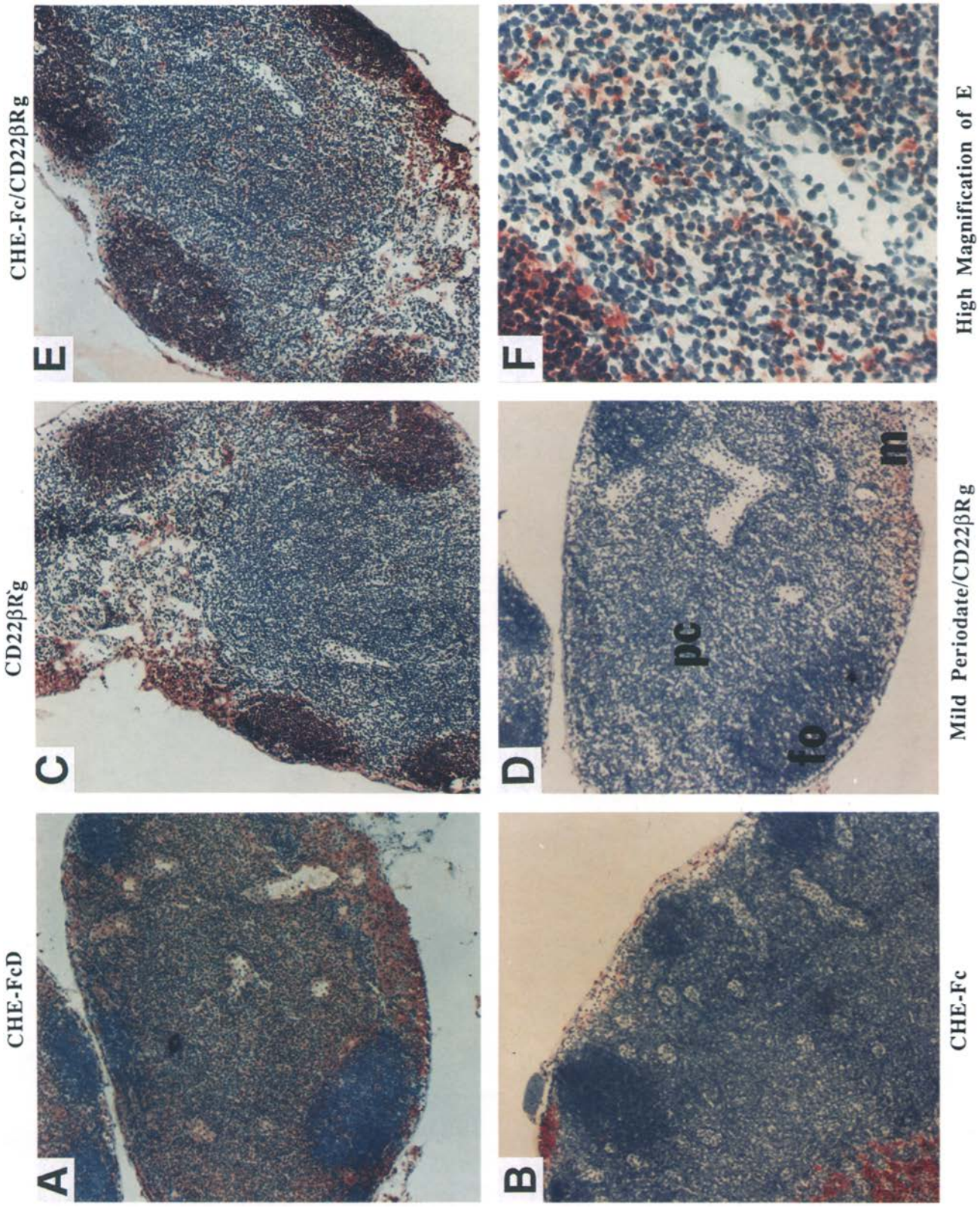

Figure 9. Immunohistological analysis of murine lymph node. Frozen sections from murine lymph node were fixed and stained as described in Materials and Methods. (*A*) x50 CHE-FcD, (*B*) x50 CHE-Fc, negative control, (*C*) x50 CD22βRg, (*D*) x50 mild periodate treatment before CD22βRg staining, (*E*) x50 CHE-Fc treatment before CD22βRg, (*F*) x500 magnification of *E* showing enhancement of dendritic cell–specific CD22βRg staining in the paracortical region. Typical examples of follicles (fo), paracortical zone (pc), and medulla (m) are indicated in *D*.

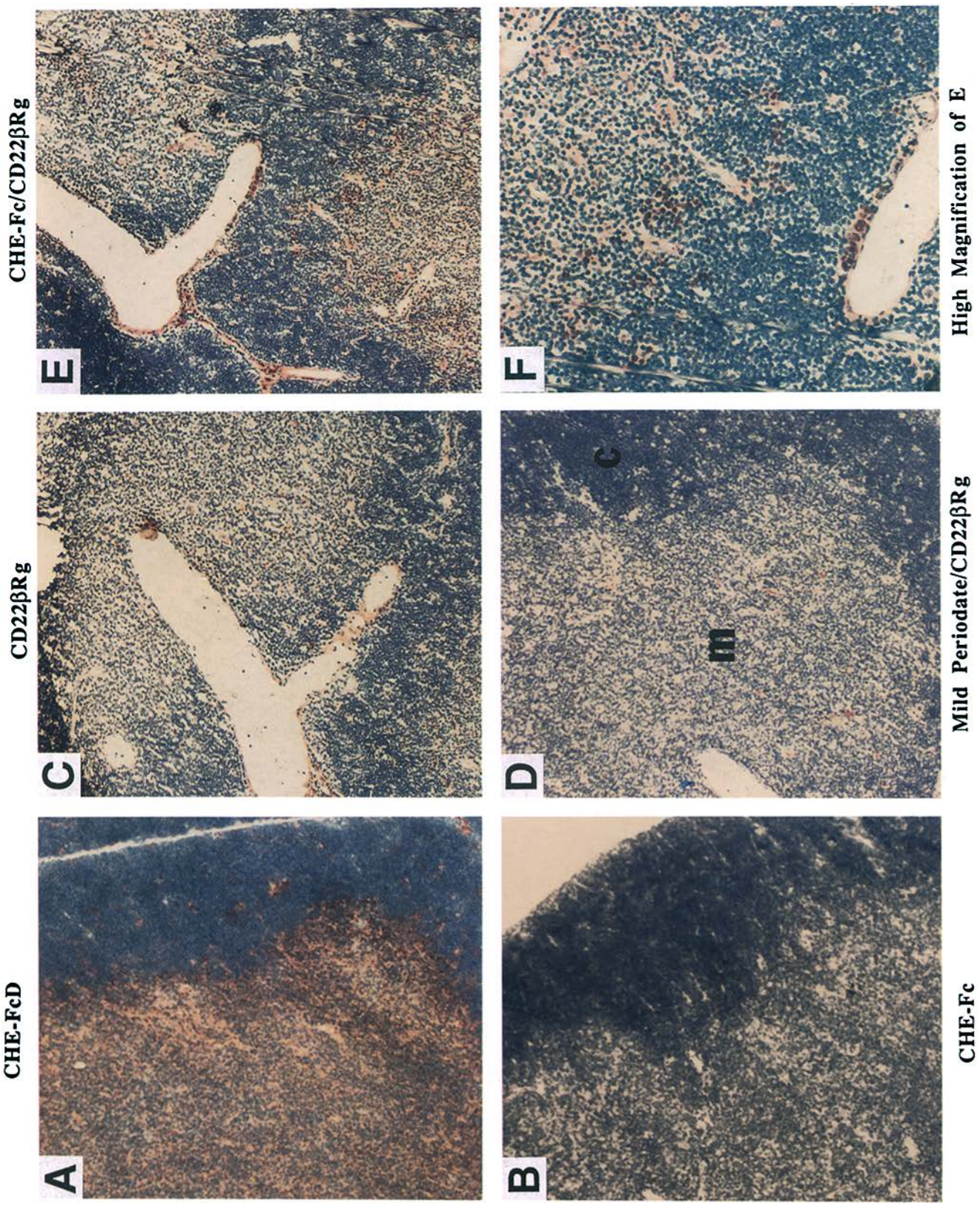

Figure 10. Immunohistological analysis of murine thymus. Frozen sections from murine thymus were fixed and stained as described in Materials and Methods. (*A*) x125 CHE-FcD, (*B*) x125 CHE-Fc, negative control, (*C*) x50 CD22βRg, (*D*) x50 mild periodate treatment before to CD22βRg staining, (*E*) x50 CHE-Fc treatment before CD22βRg, (*F*) x250 magnification of *E* showing cortical-medullary junction. Typical examples of the medulla (m) and cortex (c) are shown in *D*.

lymph node displays inverse patterns with that of CD22βRg ligands. CD22βRg strongly stains peripheral follicles of lymph nodes (enriched in B cells), while the paracortical regions are essentially negative (Fig. 9 *C*). This staining is specific, since mild periodate pretreatment abrogates it in all areas except the nonspecifically stained hilum (Fig. 9 *D*). Since the follicles stain so strongly with CD22βRg, enhancement with CHE-Fc treatment alone would be difficult to observe. However, CD22βRg staining of follicles was not restored by CHE-Fc after abrogation by mild periodate treatment, indicating that 9-*O*-acetylation does not significantly mask ligands for CD22β on these B cells (data not shown). Staining of paracortical lymphocytes (T cell–enriched region) with CD22βRg was minimal. However, if the colorimetric reaction was allowed to proceed for a longer time, specific reactivity was observed, suggesting a low level of native ligand expression (data not shown). Unlike the case in the follicles, treatment with the CHE-Fc esterase strikingly increased CD22βRg staining in the paracortical region (Fig. 9 *E*). At higher magnifications, this enhanced staining corresponds mainly to cells interspersed between lymphocytes (interdigitating dendritic cells) rather than with the perinuclear pattern typical of lymphocyte staining (Fig. 9 *F*). Interestingly, essentially all the CD22β ligands expressed by these accessory cells are masked by 9-*O*-acetylation, suggesting that this modification may regulate interactions with CD22-positive B cells.

CD22βRg Selectively Stains the Thymic Medulla and Staining Is Enhanced by Removal of 9-O-Acetyl Esters

Expression of total 9-*O*-acetylated sialic acids (CHE-FcD staining) is restricted to the medullary region of thymic tissue sections (Fig. 10 *A*), and the specificity control with CHE-Fc is negative (Fig. 10 *B*). At higher magnifications, the medullary staining is seen to be on both thymocytes and epithelial cells (data not shown). CD22βRg ligands are expressed at low levels almost exclusively within the medulla (Fig. 10 *C*), qualitatively paralleling that of total 9-*O*-acetylated sialic acids. Pretreatment with mild periodate abolished CD22βRg reactivity, indicating specificity of the staining (Fig. 10 *D*). Medullary reactivity is significantly enhanced by previous removal of 9-*O*-acetyl esters with CHE-Fc (Fig. 10 *E*). At higher magnification, it is evident that this enhancement is particularly on a subset of thymocytes at the cortical–medullary junction and on accessory cells scattered throughout the medulla (Fig. 10 *F*). Also, some cells surrounding blood vessels (possibly mast cells) stain strongly for CD22βRg only after removal of 9-*O*-acetyl esters. As before, the enhancement of CD22βRg staining by CHE-Fc treatment is specific for its esterase activity because no staining is observed when sections are treated with CHE-Fc alone (Fig. 10 *B*). Thus, medullary thymocytes, particularly at the cortical-medullary junction, as well as some medullary accessory cells, express ligands for CD22β that are masked by 9-*O*-acetylation.

Discussion

Previous reports using CD22βRg as a probe to detect ligands were limited to T and B lymphoma cell lines and to human peripheral blood T cells (2, 34, 39). Supporting the functional relevance of these findings, reactivity with CD22βRg correlated with adhesion of cells to CD22β-transfected COS cells. Antibodies directed against CD22 itself have shown that its expression is mainly on primary and secondary follicular B cells. In contrast, interfollicular regions within the medulla and paracortical regions of lymph nodes were not reactive (7). Staining of mantle zone B cells was more prominent than on germinal center B cells, suggesting that CD22 surface expression is lost after B cell activation. In this study, we have reported the direct detection of potential ligands for CD22 in lymphoid tissues and their masking by 9-*O*-acetylation of sialic acids.

In most previously studied interactions between lymphoid cells, the receptor/ligand pairs have extracellular integrin-like or Ig-like domains that mediate adhesion through protein–protein interactions (37). CD22β contains seven Ig domains and can mediate adhesion to a variety of hematopoietic cells (8, 38, 39). The recombinant chimeric form CD22βRg contains the three Ig domains required for cell adhesion, and precipitates several glycoproteins from labeled cells, including CD45RO, a protein tyrosine phosphatase (2, 43). However, CD22β mediates adhesion to CD45RO-negative cells as well (8, 27, 34, 39). This and other data indicate that CD22β interacts with a variety of distinct glycoprotein ligands. All such interactions studied to date are abolished by sialidase treatment or by mild periodate oxidation (2, 8, 27, 34, 39), which can selectively cleave the side chain of sialic acids, even on intact cell surfaces. The critical role of α2-6–linked sialic acids in these interactions (27, 28) is further confirmed here by the effects of 9-*O*-acetylation, a natural modification.

The α2-6 STN transfers sialic acids from CMP-sialic to Galβ1-4GlcNAc sequences commonly found at the nonreducing termini of N-linked oligosaccharides (48). The number and proportion of such residues depends on many factors, including the level of Golgi α2-6 STN, competing terminal transferases, the Golgi localization of α2-6 STN relative to competing enzymes, the availability of CMP-sialic acid in the Golgi lumen, the nature and amount of the polypeptide acceptors available, and their rate of transit through the Golgi. Regardless, oligosaccharides with multiple α2-6 sialic acids are relatively common on glycoproteins (14). While this may explain the multiplicity of the natural glycoprotein ligands recognized by CD22β, it does not explain why the great majority of cellular glycoproteins are not good ligands (27, 42). Thus, additional mechanisms must exist by which CD22β-glycoprotein interactions are regulated. Several possibilities exist that are not mutually exclusive. First, CD22β may recognize not only α2-6–sialylated oligosaccharides, but also a certain conformation or sequence in the underlying protein. Second, the protein may act as a "scaffold" to present the oligosaccharides in a specific pattern that is best recognized by CD22β. Third, high affinity ligands for CD22β may simply contain several N-linked oligosaccharides with α2-6–linked sialic acids. Finally, the α2-6–linked sialic acids might be modified to temporarily or permanently abolish interactions. This manuscript provides evidence for the last possibility.

Since the exocyclic side chain of sialic acid is known to be required for recognition, it is satisfying to find that side-chain 9-*O*-acetylation can abrogate recognition of α2-6–linked sialic acids by CD22β. Interestingly, solution con-

formation studies of sialyl α2-6*N*-acetyl-lactosamine by ^{1}H-NMR indicate that the sialic acid moiety folds back on the underlying oligosaccharide backbone because of flexibility of the glycosidic linkage between galactose and sialic acid. This causes the side chain of sialic acid to protrude into space away from the oligosaccharide backbone(4, 30), perhaps allowing access for recognition by CD22β. Although the solution conformation of 9-*O*-acetylated sialyl α2-6*N*-acetyl-lactosamine has not been solved, it is easy to conceive that addition of a hydrophobic, bulky acetyl ester to the C9 hydroxyl group would disrupt the CD22β interaction.

We also show here that 9-*O*-acetylation is in a position to modulate CD22β interactions in lymphoid tissues in vivo. In the spleen, B cells are the predominant class of cells expressing masked (9-*O*-acetylated) ligands for CD22βRg. In contrast to splenic B cells, lymph node–associated follicular B cells expressed CD22β ligands, none of which were masked by 9-*O*-acetylation. Thus, masking of CD22β ligands by 9-*O*-acetylation is differentially regulated on the same cell type between two different lymphoid organs.

When CHE-FcD was used to detect total 9-*O*-acetylated sialic acids, the reactivity was more widespread, and did not follow the same pattern as that of CD22β ligands. Thus, most of the 9-*O*-acetyl esters on splenic and lymph node–derived cells are on molecules other than CD22β ligands. In keeping with this, although most T cells expressed only low levels of *O*-acetylated CD22β ligands, many had high levels of total 9-*O*-acetylated sialic acids. These are presumably on other *O*-acetylated molecules, such as CDw60, which has recently been shown to be related to the ganglioside 9-*O*-acetylated G_{D3} (18, 19). This also supports our previous work indicating that different cell types express *O*-acetylation of sialic acids only on specific classes of glycoconjugates (5, 21), and that even within these classes, it is expressed only on specific sialic acid residues (35). Thus, 9-*O*-acetylation of CD22β ligands is unlikely to by a nonspecific consequence of general *O*-acetylation of sialic acids in the cell type in question. Rather, it is likely to be a regulated event, presumably meant to fulfill some modulatory function on CD22β-mediated biology. The adhesion assays presented here indicate that CD22β-dependent intercellular adhesion may be one of these functions. Further analysis will be required to determine the effect of masking CD22β ligands on the process of B cell activation. Within the thymus, low but distinct expression of CD22β ligands was seen almost exclusively in medullary thymocytes and epithelial cells. Since medullary thymocytes are mature relative to cortical thymocytes, this indicates that CD22β ligands are induced as thymocytes mature. Treatment with CHE-Fc greatly enhanced this staining, particularly on a subset of thymocytes at the cortical–medullary junction.

The primary goal of this work was to see if 9-*O*-acetylation could affect the binding of CD22β to either synthetic or natural ligands. A complete analysis of the phenotypes of all the different subsets of cells in the lymphoid organs in relation to CD22β ligand expression is needed in the future. However, since different cell types are segregated into distinct microdomains within lymphoid tissues (10, 24, 46), some further conclusions and speculations can be made. Although specific accessory cell markers were not used in this study, CD22βRg staining of these cells was identifiable by their characteristic staining in an interdigitating pattern between lymphocytes, particularly locations within the tissues (6, 10, 24, 46). In all of the lymphoid tissues, accessory cell staining with CD22βRg was significantly enhanced by removal of 9-*O*-acetyl esters. In the spleen and thymus, this masking is partial, whereas essentially all the ligands on interdigitating dendritic cells of the lymph node are masked. Since CD22β may function both as an adhesion protein and as an activation molecule (25, 26), immune cells may negatively regulate these events by inducing the expression of 9-*O*-acetyl esters on CD22β ligands. For example, the cross-linking of CD22β by interaction with ligands on accessory cells could facilitate B cell activation, as well as a response by the accessory cells. If so, the expression of 9-*O*-acetyl esters would negatively regulate these initial activation events (6, 20, 32). In particular, the extensive masking of ligands on paracortical dendritic cells of the lymph node could prevent inadvertent interactions between these cells and naive B cells expressing CD22β, which have to migrate through the paracortical region before follicular localization (24, 46, 50). Also, if thymocytes within the cortical–medullary junction are those just preparing to enter the circulation, the masking of CD22β ligands by 9-*O*-acetylation may be beneficial to avoid interactions with naive CD22-positive B cells before encountering antigens presented by accessory cells. Finally, the molecular mechanisms by which lymphocytes within lymphoid tissues are segregated into distinct microdomains (10, 46) are currently unknown. If interactions between CD22β and its α2-6–sialylated counterreceptors on T or B lymphocytes help to generate follicular morphology (49), 9-*O*-acetylation could modulate the entry of lymphocytes into this domain. A detailed study of 9-*O*-acetylation of CD22β ligands during the ontogeny of B and T cells is needed to address these speculations.

Since CD22β mediates adhesion by recognition of α2-6–linked sialylated oligosaccharides, immune cells may modulate interactions by coordinating the expression of α2-6 STN and by 9-*O*-acetylation of its product. The distribution of CD75, CD76, and HB6 (antibody epitopes recognized in a α2-6–linked sialic acid dependent manner) indicates that α2-6 STN is expressed to some extent in B cells of varied maturation states (3). However, studies of α2-6 STN RNA and of the enzyme protein (1, 9, 47) indicate that α2-6 STN expression in lymphocytes is extensively regulated. While the regulation of T lymphocyte–associated α2-6 STN expression has not been analyzed carefully, activation with phytohemagglutinin causes induction of CD22β ligands on these cells (39). Of course, this could represent expression of the correct glycoprotein acceptors rather than upregulation of the α2-6 STN. Alternatively, other transferases, such as Galβ1-4GlcNAc:α2-3 STN (48), which compete for the same substrate as α2-6 STN (but confer no interaction with CD22β), may decrease, thereby upregulating ligands for CD22β. The current work demonstrates that addition of 9-*O*-acetyl esters is another potential mechanism to alter cell phenotypes from high affinity to low affinity states for recognition by CD22β-positive B cells. Very little is known about the regulation of 9-*O*-acetylation of sialic acids on lymphocytes. In other systems, *O*-acetyl esters on sialic acids are generated by transfer of acetate, donated from cytosolic acetyl CoA to the C7 and C9 hydroxyl groups of terminal sialic acids on glycoconjugates within the lumen of the Golgi apparatus or Golgi-like elements (36, 44). When 7-*O*-acetyl es-

ters are exposed to extracellular physiologic pH, they spontaneously migrate to the more stable position at the C9 hydroxyl (16). Thus, in the case of CD22β-mediated interactions, 7-*O*-acetyl esters are unlikely to be of practical consequence.

Although our study has used the human form of CD22β, the murine equivalent is >70% homologous (41), and it interacts with ligands in a sialic acid–dependent manner (Stamenkovic, I., personal communication). We therefore chose to first study the lymphoid system of a defined murine strain. Further work will determine if 9-*O*-acetylation masks CD22β ligands in human lymphoid tissues as well. Although *O*-acetylation is generally thought to be more prevalent in rodents, 9-*O*-acetylated sialic acids have been reported on human lymphocytes (15) and on certain malignant human tumors (44). Interestingly, lymphocytes from humans with certain malignancies express markedly elevated levels of 9-*O*-acetylated sialic acids (13, 40). If these increases of 9-*O*-acetylation are occurring on lymphocyte-associated sialylated ligands for CD22β, they could negatively modulate the role of CD22β in immune responses, generating a mechanism for tumor cells to evade immune surveillance.

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