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CD22-mediated Cell Adhesion to Cytokine-activated Human Endothelial Cells

POSITIVE AND NEGATIVE REGULATION BY α2–6-SIALYLATION OF CELLULAR GLYCOPROTEINS*

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We previously showed that cultured human umbilical vein endothelial cells (HEC) exposed to the inflammatory cytokines tumor necrosis factor-α or interleukin-1 display increased activity of β-galactoside α2,6-sialyltransferase. This is associated with enhanced expression of ligands for the B cell receptor CD22β, which recognizes α2–6-linked sialic acids (Hanasaki, K., Varki, A., Stamenkovic, I., and Bevilacqua, M. P. (1994) *J. Biol. Chem.* 269, 10637–10643). Here we report that increased expression of CD22 ligands is a feature of dermal microvascular endothelial cells as well, and is also observed in response to the cytokine interleukin-4. Tumor necrosis factor-α stimulation of HEC causes no change in the profile of endothelial glycoproteins recognized by CD22, but doubles the proportion of total cellular *N*-linked oligosaccharides capable of binding tightly to CD22. This modest change is sufficient to cause a marked increase in α2–6-linked sialic acid-dependent binding of Chinese hamster ovary (CHO) cells expressing recombinant human CD22. In contrast, B lymphoma cell lines expressing higher levels of cell surface CD22 do not show such sialic acid-dependent binding to activated HEC. Since B lymphoma cells themselves also express high levels of α2–6-linked sialic acids, their CD22 molecules might be rendered nonfunctional by endogenous ligands. In support of this, the lectin function of CD22 can be directly detected on transfected CHO cells, but not on B lymphoma cells. Furthermore, coexpression of β-galactoside α2,6-sialyltransferase with CD22 in the CHO cells abrogates sialic acid-dependent binding to cytokine-activated HEC. However, such co-transfected cells can bind to B lymphoma cells in a manner apparently less dependent upon α2–6-linked sialic acid, suggesting CD22-mediated interactions that may not be directly dependent on its lectin function. Thus, CD22-mediated interactions between B cells and activated vascular endothelium may be positively regulated by induction of α2–6-linked sialic acid-bearing endothelial cell ligands, but negatively regulated by such ligands on the B cells expressing CD22. Since expression of both CD22 and β-galactoside α2,6-sialyltransferase are regulated during B cell ontogeny, these findings could be of importance in B cell function and/or trafficking.

Sialic acids (Sias)[1] are a family of nine-carbon carboxylated sugars found at terminal positions of mammalian cell surface sugar chains (1, 2). Because of their location and negative charge, Sias can inhibit cell-cell interactions by nonspecific (2, 3) or specific (4) mechanisms. However, they can also serve as ligands for specific cell-cell recognition molecules such as the selectins, sialoadhesin, and CD22 (5–12). CD22 is a B cell-restricted glycoprotein whose sequence defines it as a member of the immunoglobulin (Ig) superfamily. CD22β is the larger of two human CD22 isoforms identified, containing two additional Ig domains (numbers 3 and 4) not present in the shorter isoform CD22α (13). Early studies indicated that CD22 may function in B cell activation (14, 15), and its cytoplasmic domain becomes rapidly phosphorylated following B cell stimulation with anti-μ (16). Additionally, CD22 can function as an adhesion molecule, mediating interactions with activated blood cells and accessory cells (13, 17–19). A soluble chimeric form of CD22β (CD22Rg), containing the three amino-terminal Ig-like domains of human CD22β fused to the COOH-terminal Fc domains of human IgG can bind and precipitate potential glycoprotein ligands on activated T and B cells, one of which is the tyrosine phosphatase CD45 (9, 13, 20, 21). Sialic acid (Sia) is an essential component of recognition by CD22 (13). Several studies have established that the structural motif specifically recognized is Siaα2–6Galβ1–4GlcNAc- (9, 13, 19, 21) (see also the accompanying paper (22)), which is found in varying numbers on the antennae of *N*-linked oligosaccharides of some cell surface glycoproteins (1, 23). In contrast, α2–3-linked Sia-containing structures found on the same oligosaccharides are not recognized.

Many different sialyltransferases have been identified that can transfer Sia residues onto glycoprotein oligosaccharides (23, 24). Each shows specificity not only for the linkage formed (α2–3, α2–6, or α2–8), but also for the acceptor structure. The enzyme responsible for synthesizing the structure recognized by CD22 is β-galactoside α2,6-sialyltransferase (ST6N), which catalyzes the reaction: CMP-Sia + Galβ1–4GlcNAcβ1- → CMP + Siaα2–6Galβ1–4GlcNAcβ1- (25, 26). ST6N expression is increased in the liver during inflammatory responses and

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[1] The abbreviations used are: Sia, sialic acids; AGP, α_1-acid glycoprotein; CD22Rg, CD22-immunoglobulin chimera containing Ig domains 1–3 of human CD22β; FCS, fetal calf serum; CD22Rg-PAS, CD22Rg coupled to protein A-Sepharose; HEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; Ig, immunoglobulin; IL-1, interleukin-1; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PE, phycoerythrin; sLac, sialyllactose; SNA, *Sambucus nigra* agglutinin; ST6N, β-galactoside α2,6-sialyltransferase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; WT, wild-type.

shows a regulated expression in other tissues (24, 27, 28). This results at least in part from the activity of different 5′ promoter elements, including a lymphocyte-specific promotor (29). Some cell surface epitopes (*e.g.* CD75 and CD76) defined by monoclonal antibodies which demonstrate specific histological patterns in lymphoid tissues have also been shown to depend upon the presence of α2–6-Sias and the expression of ST6N (30–34).

Activation of vascular endothelium by cytokines and bacterial products results in a coordinated display of new cell-surface glycoproteins including the cell adhesion molecules E-selectin, ICAM-1, and VCAM-1 (35–37). We have previously shown that inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), as well as lipopolysaccharide (LPS), act on human umbilical vein endothelial cells (HEC) to induce increased expression of cellular ST6N (38). This is accompanied with enhanced expression of α2–6-linked Sias (detected by *Sambucus nigra* agglutinin, SNA) and an increase of CD22 ligands (detected by α2–6-linked Sia-dependent binding of soluble CD22Rg) (38). Here, we explore if this increased binding of CD22Rg to cytokine-activated HEC is mediated by newly synthesized glycoproteins or by an increase in CD22 binding to *N*-linked oligosaccharides. Also, while CD22-dependent cell adhesion events involving cytokine-activated HEC can be positively regulated by induction of α2–6-linked Sia-bearing endothelial cell ligands, we demonstrate negative regulation of CD22 by α2–6-sialylated ligands on B cells expressing CD22. The latter fits well with the recent work of Braesch-Andersen and Stamenkovic (39), who showed that CD22 molecules bearing α2–6-linked Sia are functionally inactive.

EXPERIMENTAL PROCEDURES

Materials—Development and characterization of a soluble CD22Rg containing the three amino-terminal Ig-like domains of human CD22 fused to the COOH-terminal Fc domains of human or murine IgG, and of P-selectin-Rg has been described previously (9, 21). All experiments described here were performed with the CD22Rg construct bearing the human Ig Fc tail, except for the staining of HEC cells, which employed the CD22Rg construct with a murine Ig Fc tail. The following reagents and chemicals were obtained from the commercial sources indicated: α2–3- and α2–6-sialyllactose (sLac), Oxford Glycosystems Inc. (New York); aprotinin, bovine serum albumin (BSA), glutaraldehyde, human IgG_1, *o*-phenylenediamine, phenylmethylsulfonyl fluoride, and porcine heparin, Sigma; protein A-Sepharose, Pharmacia Biotech Inc.; peroxidase-conjugated goat anti-mouse IgG antibody, Bio-Rad; phycoerythrin (PE)-conjugated mouse anti-human CD22 monoclonal antibody (mAb) BL22, Caltag Laboratories (San Francisco, CA); PE-conjugated mouse anti-human CD22 mAb Leu-14 and endothelial cell growth supplement, Becton Dickinson. (Oxnard, CA); FITC-conjugated SNA, E. Y. Laboratories, Inc. (San Mateo, CA); and IL-4, Genzyme (Cambridge, MA). Human recombinant TNF-α was a gift from Biogen Corp. (Cambridge, MA). Anti-VCAM-1 mAb E1/6 (IgG_1; ammonium sulfate-precipitated Ig) (40, 41) was obtained from Dr. M. P. Bevilacqua (Amgen Inc., Thousand Oaks, CA). cDNAs encoding human ST6N cDNA and the human membrane form of CD22 were gifts from Dr. I. Stamenkovic, MGH, Boston, MA. Protein concentrations were determined using the bicinchonic acid protein assay reagent kit (Pierce) with BSA as a standard.

Endothelial Cell Culture and Stimulation—Primary cultures of HEC from Clonetics Corp. (San Diego, CA) were grown in dishes coated with 0.1% gelatin using Medium 199 (Irvine Scientific) with 20% fetal calf serum (FCS), 50 μg/ml endothelial cell growth supplement, and 100 μg/ml porcine heparin and subcultured using trypsin/versene. Microvascular endothelial cells isolated from normal human neonatal foreskin capillary vessels were from Cell Applications, Inc. (San Diego, CA), and were cultured in the proprietary CADMEC Growth Medium supplied by the manufacturer. Confluent endothelial cells (passage 2–3) were typically activated by incubation at 37 °C for 48–72 h with 200 units/ml TNF-α or 10 ng/ml IL-4.

Sialyltransferase Assay—After stimulation of HEC in 100-mm diameter dishes with IL-4 (10 ng/ml) for 48 h, cells were lysed and the cell-associated sialyltransferase activity was assayed as described previously (38).

Assay for CD22Rg Binding to HEC—Confluent endothelial cells grown in 96-well plates were stimulated for 48 h. After washing three times with Medium 199, cells were incubated with 2.5 μg/ml CD22Rg in Medium 199, 1% BSA at 4 °C for 2 h. After washing, cells were incubated with peroxidase-conjugated goat anti-mouse IgG Ab at 4 °C for 1 h. The plates were washed 3 times with Hank's balanced salt solution followed by the addition of 0.5 mg/ml *o*-phenylenediamine in 50 mM sodium citrate, 50 mM sodium phosphate buffer, pH 5.3, containing 0.01% (v/v) H_2O_2. The reaction was stopped by the addition of 4 N H_2SO_4, and the plates were read at 492 nm. Results are expressed as A_{492} values after subtracting the background obtained with secondary Ab alone.

Affinity Precipitation of Endothelial Ligands for CD22—Confluent HEC in 35-mm dishes were grown in complete medium supplemented with 34 μCi/ml [6-^{3}H]glucosamine (DuPont NEN) for 72 h with or without stimulation with 200 units/ml TNF-α. After extensively washing with ice-cold phosphate-buffered saline (PBS, pH 7), the labeled cells were solubilized in lysis buffer (PBS containing 0.5% Nonidet P-40, 0.02% sodium azide, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatants were collected. HEC lysates were precleared with human IgG_1 (5 μg/ml) and 20 μl of packed protein A-Sepharose beads by incubation for 4 h at 4 °C. Precleared lysates were incubated with 15 μl of fresh protein A-Sepharose beads and 6 μg of CD22Rg or P-selectin-Rg protein for 4 h at 4 °C. Beads were washed 5 times in lysis buffer and precipitates were eluted by boiling for 5 min in Laemmli sample buffer in the presence of 5% 2-mercaptoethanol. Samples were analyzed by SDS-polyacryamide gel electrophoresis using 4–20% acrylamide gradient gels, followed by fluorography using Intensify (DuPont NEN).

Analysis of CD22 Binding Activity of N-linked Oligosaccharides Isolated from HEC by CD22Rg Column Chromatography—Confluent HEC were grown in complete medium supplemented with 16 μCi/ml [6-^{3}H]glucosamine for 72 h with or without stimulation with 200 units/ml TNF-α. After washing extensively with PBS, the labeled cells were solubilized in lysis buffer (4% (w/v) SDS, 20 mM 2-mercaptoethanol, 10 mM HEPES, pH 7.0) by heating to 80 °C for 20 min. Isolation of ^{3}H-labeled *N*-linked oligosaccharides from the glycoproteins in the lysates was performed as described (38), using peptide *N*-glycosidase F. The radioactivity incorporated into isolated *N*-linked oligosaccharides from the glycoproteins were 1139 ± 34 cpm/μg of protein in resting HEC and 968 ± 28 cpm/μg of protein in TNF-α-activated HEC (n = 3).

The CD22 binding activity of oligosaccharides was performed as described previously (21, 42, 43). Briefly, CD22Rg columns were constructed by adsorbing 1 mg of CD22Rg to 0.75 ml of protein A-Sepharose in a siliconized 1-ml polystyrene pipette. Samples of ^{3}H-labeled oligosaccharides (originating from 18 μg of endothelial cell protein) were mixed with the non-binding marker [^{14}C]ManNAc, and then applied to the column at 4 °C in Tris-buffered saline (20 mM Tris-HCl, pH 7.3, 140 mM NaCl, 0.02% sodium azide). Three drop fractions (about 80 μl) were collected for 19 fractions (as indicated in the text), the column warmed to room temperature for 15 min and then eluted for a further 21 fractions with buffer at room temperature.

Cell lines and Transfection—B cell lymphoma lines (Daudi and Raji) were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, streptomycin, and penicillin. CHO cells were cultured in α-MEM supplemented with 10% FCS, L-glutamine, streptomycin, and penicillin. CHO cells were transfected with 0.5 μg of G418 PU cDNA and 10 μg of a plasmid containing full-length human CD22β cDNA and/or ST6N cDNA with a cytomegalovirus promoter using the calcium-phosphate method (44). After 7–10 days of selection in G418 (1 mg/ml active concentration), individual clones isolated by the direct transfer of individual colonies to a 96-well microtiter tray. Expression of transfected proteins was determined by flow cytometry employing either FITC-conjugated SNA (recognizes the sequence Siaα2–6Galβ1–4GlcNAc) in the absence or presence of 0.2 M lactose, or PE-conjugated BL22. Clones expressing differing levels of CD22 and/or ST6N were selected and cultured in α-MEM, 10% FCS medium containing 1 mg/ml G418. In parallel, another set of CHO cells were transfected with just the CD22-containing plasmid along with G418 PU cDNA, and selected as above.

Flow Cytometry Analysis—Transfected CHO cells were harvested from dishes by incubation with 0.53 mM EDTA/PBS for 10 min at 37 °C. Cells (1 × 10^6) were washed twice in PBS and incubated with PE-conjugated BL22 (4 μg/ml) or PE-conjugated Leu-14 (2 μg/ml) (anti-CD22 mAbs), or FITC-conjugated SNA (20 μg/ml) in 100 μl of PBS, 0.1% sodium azide, 0.1% BSA. For probing of the lectin function of CD22 on cell surfaces, FITC-tagged $α_1$-acid glycoprotein (AGP) was prepared previously (42), utilizing a preparation of AGP containing 7 and 12

mol/mol protein of α2–3- and α2–6-linked Sia, respectively (other lots of AGP were found to contain lower amounts of sialylation and were not suitable for flow cytometry). After incubation for 40 min at room temperature, cells were washed with PBS, 0.1% sodium azide, 0.1% BSA and fixed in PBS containing 1% formaldehyde. Stained fixed cells were analyzed on a FACscan® instrument (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Cell Adhesion Assay with HEC—Confluent HEC in 96-well plates were stimulated for 48 h with TNF-α as described above. Before assay, HEC monolayers were washed three times with assay medium (RPMI 1640 containing 1% BSA) and incubated with 50 μl of assay medium alone or medium containing anti-VCAM-1 mAb E1/6 (20 μg/ml) for 20 min at 4 °C. In parallel, EDTA-harvested CHO cells or B cell lymphomas were washed three times and resuspended with ice-cold assay medium. The cell suspensions (50 μl) were then added to HEC monolayers and incubated for 30 min at 4 °C. After washing three times with the assay medium to remove unbound cells, adherent cells were fixed with ice-cold 2.5% glutaraldehyde/PBS and counted microscopically.

Adhesion Assay of Transfected CHO Cells to Daudi Cells—CHO and transfected cells were trypsinized and transferred to a 35-mm dish at a density of 2×10^5 cells/dish, and cultured for 48 h. Before assay, these cells were washed 3 times with ice-cold DMEM. In parallel, Daudi cells were washed and resuspended in DMEM. Daudi cell suspension was added to transfected CHO cells in the dish (2×10^6 cells/dish) and incubated for 30 min at 4 °C. Cells that did not bind to CHO cells were removed by washing 4 times with ice-cold DMEM and bound cells were fixed in DMEM containing 2% formaldehyde. The binding of Daudi cells was quantitated by counting the total number of cells bound to 200 CHO cells.

For some blocking experiments with α2–6-sialyllactose (α2–6-sLac), an alternative quantitative assay was performed. Transfected CHO cells were cultured in a 48-well plate to confluency, and washed three times with PBS before assay. Daudi cells (2×10^6/ml) were cultured in RPMI, 10% FCS supplemented with 5 μCi/ml [*methyl*-^{3}H]thymidine (ICN Biomedicals, Inc.) for 48 h. After washing extensively with PBS, 2 mM EDTA, 1% BSA, ^{3}H-labeled Daudi cells were resuspended in PBS, 0.5% BSA, 2 mM EDTA, 4 mM $MgCl_2$. Daudi cell suspensions were mixed with various concentrations of α2–6-sLac, added to the CHO cells and incubated under rotation (100 rpm) for 30 min at 4 °C. After washing 4 times with ice-cold PBS, the bound cells were solubilized with 0.1% Nonidet P-40/PBS, and their radioactivity was counted.

RESULTS

Increased CD22 Ligand Expression Is Seen with IL-4 Activation and in Microvascular Endothelial Cells—TNF-α, IL-1, or LPS each cause increased expression of ST6N in HEC (38). This is accompanied by a small increase in α2–6 linkage of total Sias on endogenous *N*-linked oligosaccharides, and an enhanced expression (2–4-fold) of ligands for the B cell adhesion molecule CD22 (measured with the soluble immunoglobulin chimera CD22Rg) (38). While TNF-α, IL-1, or LPS are known to increase surface expression of VCAM-1, ICAM-1, and E-selectin (37), another cytokine IL-4 is known to selectively activate HEC to induce only VCAM-1 expression (45). We found that treatment of HEC with IL-4 (10 ng/ml) for 48 h induces a 2.5-fold increase in cell-associated sialyltransferase activity as well as a 2-fold increase in CD22Rg binding (data not shown). Thus, a variety of cytokines can induce HEC cells to express both ST6N and potential ligands for CD22.

HEC are commonly used model cells for the study of endothelial cell biology. To establish that the biological responses we observed are a more general feature of vascular endothelial cells, we examined microvascular endothelial cells derived from human neonatal foreskin. As shown in Fig. 1, treatment of these cells with TNF-α resulted in increased CD22 ligand expression on these cells. As with HEC, induction of CD22Rg ligands is both dose- and time-dependent, with a half-maximal effect at a similar concentration (~2 units/ml) of TNF-α (38). Thus, increased CD22 ligand expression in response to cytokines is not a unique feature of human HEC, but may be a general feature of endothelial cell activation.

Increase in CD22Rg Binding to Activated HEC Is Not Explained by Expression of New Glycoprotein Ligands—To identify the major CD22 ligands, the endogenous glycoproteins of quiescent and TNF-α-activated HEC were metabolically labeled with [6-^{3}H]glucosamine and affinity purified with CD22Rg coupled to protein A-Sepharose (CD22Rg-PAS). As shown in Fig. 2, a number of different glycoproteins of estimated molecular mass between 130 and 160 kDa, as well as some above 200 kDa, were isolated from resting HEC. No proteins were isolated with P-selectin-Rg (having the same human IgG_1 Fc tail), indicating the specificity of CD22Rg binding. Following stimulation with TNF-α for 72 h, no major changes in the profile of CD22Rg-bound molecules were seen (Fig. 2). Thus, the 2–4-fold increase in CD22Rg binding to HEC cells following TNF-α stimulation as demonstrated previously (38) does not appear to be due to the selective expression of new glycoprotein ligand(s).

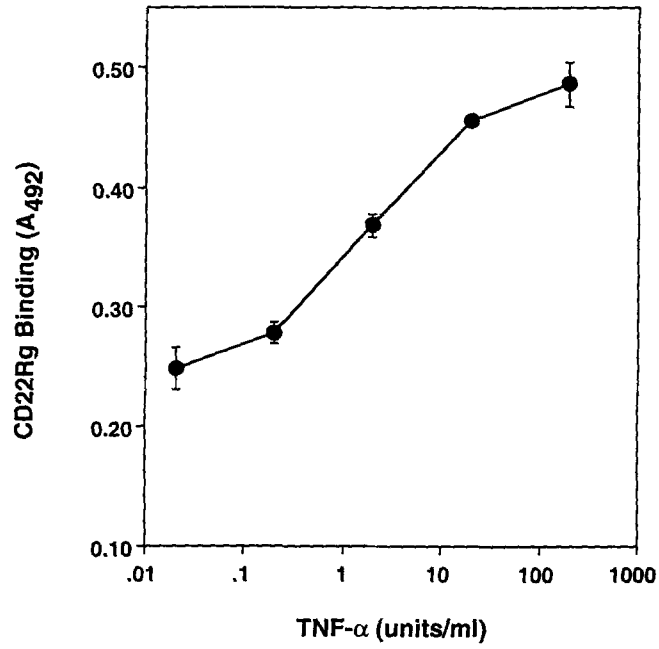

FIG. 1. **Increase in CD22Rg binding upon activation of human dermal microvascular endothelial cells with TNF-α.** Confluent microvascular endothelial cells, stimulated for 48 h with various concentrations of TNF-α as indicated, were incubated with CD22 mRg. After washing, cells were incubated with peroxidase-conjugated goat anti-mouse IgG Ab and binding was detected as described under "Experimental Procedures." The data are the mean ± S.E. of triplicate determinations.

TNF-α Activation Causes an Increase in Proportion of Total Cellular N-linked Oligosaccharides in HEC Capable of Binding to CD22Rg—Since the improved binding of CD22Rg to cytokine-activated HEC cannot be explained by the expression of novel glycoprotein ligands, we reasoned that it may be due to improved binding of *N*-linked oligosaccharides on the same proteins. To explore this possibility, ^{3}H-labeled *N*-linked oligosaccharides from total metabolically labeled glycoproteins were released with peptide *N*-glycosidase F and studied for binding to a column of CD22Rg-PAS. When applied to this column, nonbinding molecules elute in the column's V_i (coeluting with [^{14}C]ManNAc, included as an internal noninteracting marker), whereas binding molecules elute later. We have previously shown that oligosaccharides eluting 2–5 fractions past the V_i of such a column carry a single α2–6-linked Sia residue, and those eluting after warming the column to ambient temperature contain two or more α2–6-linked Sia residues (21, 42, 43). As shown in Fig. 3, both resting and stimulated HEC cells contain a significant number of *N*-linked oligosaccharides containing no (fractions 9–14) or one (fractions 15–18) α2–6-

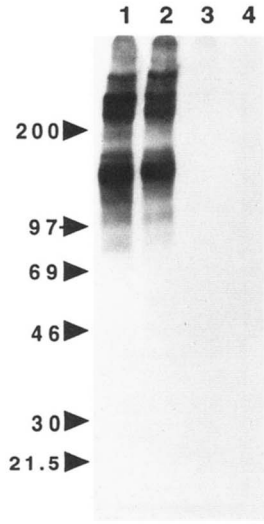

FIG. 2. **Identification of CD22-binding proteins in quiescent and cytokine-activated HEC by affinity precipitation.** HEC were incubated with or without 200 units/ml TNF-α in the medium containing [6-^{3}H]glucosamine for 72 h. The cell lysates were precipitated with CD22Rg or P-selectin-Rg and PAS. Bound proteins were resolved by SDS-polyacrylamide gel (4–20%) electrophoresis and detected by fluorography. CD22Rg precipitates of lysates derived from unstimulated (*lane 1*) and TNF-α-stimulated HEC (*lane 2*), and P-selectin-Rg precipitates derived from unstimulated (*lane 3*) and TNF-α-stimulated HEC (*lane 4*) were shown. Molecular mass markers (*kDa*) are indicated.

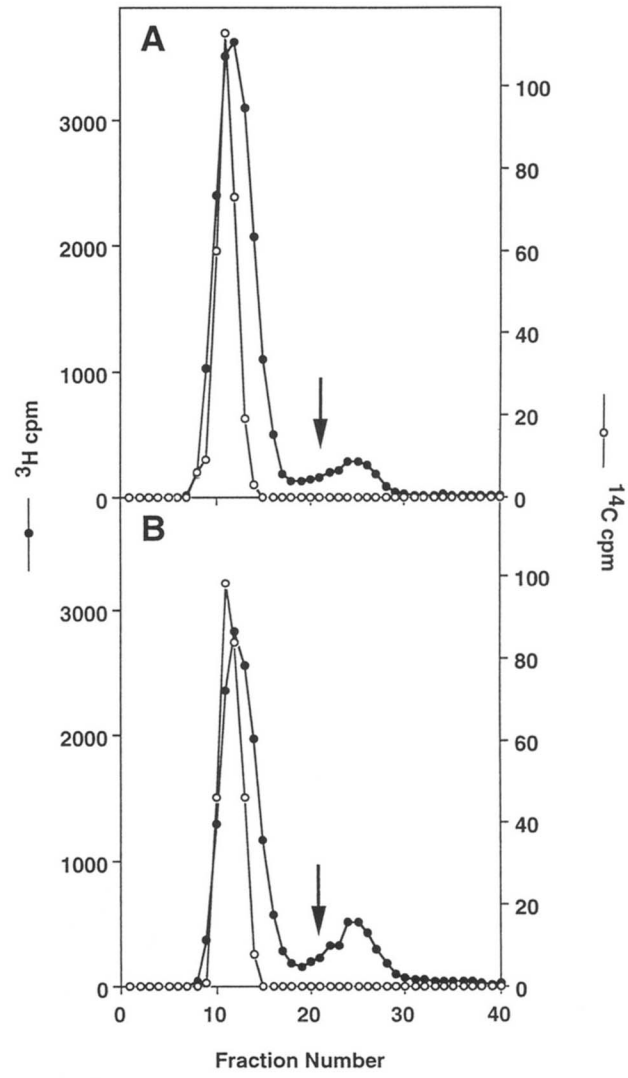

FIG. 3. **CD22Rg column affinity chromatography of *N*-linked oligosaccharides isolated from [^{3}H]glucosamine-labeled HEC glycoproteins.** HEC were incubated with or without 200 units/ml TNF-α in the medium containing [6-^{3}H]glucosamine for 72 h. *N*-Linked oligosaccharides were released from total glycoproteins using peptide *N*-glycosidase F, purified, desalted, and concentrated. Aliquots corresponding to material derived from 18 μg of protein were mixed with 300 cpm of [^{14}C]ManNAc, and applied to a CD22Rg-PAS column. Fractions (80 μl) were collected and their radioactivity monitored. The *arrow* indicates the point at which the column was warmed from 4 °C to ambient temperature. *A*, unstimulated HEC. *B*, TNF-α-stimulated HEC.

linked Sia residues. For resting cells, about 10% of total *N*-linked oligosaccharides elute in a position corresponding to two or more α2–6-linked Sia residues (fractions 20–30), while TNF-α treatment induces a 2-fold increase in this population (to 20.5%, compare Fig. 3, *A* and *B*). Thus, the increased binding of CD22Rg to activated HEC is mediated by a small but significant increase of multiply α2–6-sialylated *N*-linked oligosaccharides capable of higher affinity interactions with CD22.

CHO Cells Transfected with Full-length Human CD22 Show α2–6-Linked Sialic Acid-dependent Binding to TNF-α Activated HEC—All studies to this point were performed with a recombinant soluble form of CD22 carrying only the first three of the seven Ig-like domains of the native protein. Moreover, as shown in the accompanying paper (22) this chimera may be functionally a dimer, which may potentially have adhesion properties different from the full-length cell-associated protein. To ensure that the increase in HEC expression of ligands for this recombinant molecule correlates with improved cell adhesion through cell-surface CD22, we used CHO cells stably transfected with full-length human CD22. Wild-type CHO cells (WT-CHO) were chosen for transfection as it is known that they do not express endogenous ST6N (46, 47). As shown in Fig. 4*A*, CHO cells expressing CD22 (CD22-CHO) showed a marked increase in binding to TNF-α-activated HEC, whereas non-transfected WT-CHO cells bound to neither resting nor activated HEC. Notably, the difference in cell binding between quiescent and activated HEC is much more dramatic than predicted by the 2–4-fold increase in binding of soluble CD22Rg to activated HEC cells (38) or by the ~2-fold increase in multiply sialylated *N*-linked oligosaccharides exhibiting high affinity binding to CD22Rg in the column assay (Fig. 3). This enhanced binding is 85% inhibited by coincubation with α2–6-sLac (Fig. 4*B*). As a control, antibodies to VCAM-1 (which is known to be expressed on activated HEC cells (see Ref. 45), gave no inhibition of CD22-CHO binding to activated HEC (Fig. 4*B*).

B Lymphoma Cells with Higher Levels of Surface CD22 Bind to Activated HEC, but Binding Is Dependent upon VCAM-1, and Not on α2–6-Linked Sias—It is difficult to obtain pure populations of resting CD22-positive B cells from normal humans, free of all activated B cells and other cell types. On the other hand, there are presently no established cell lines that maintain the true phenotype of resting CD22-positive B cells. We therefore examined the binding of Daudi cells, a CD22-expressing B cell lymphoma cell line, to cytokine-activated HEC cells. By flow cytometry, these malignant B cells express somewhat higher levels of CD22 than the CD22-transfected CHO cells employed in Fig. 4 (see Fig. 5). Indeed, 4-fold more Daudi cells bound to TNF-α stimulated HEC cells than to unstimulated monolayers (see Fig. 6). However, unlike the case with the CD22-CHO cells, pretreatment with anti-VCAM-1 antibody suppressed about 80% of this interaction and α2–6-sLac showed no inhibitory effects (see Fig. 6). Thus, the Daudi cell binding largely depends on the VCAM-1 molecule expressed on the cytokine-activated HEC (presumably binding to

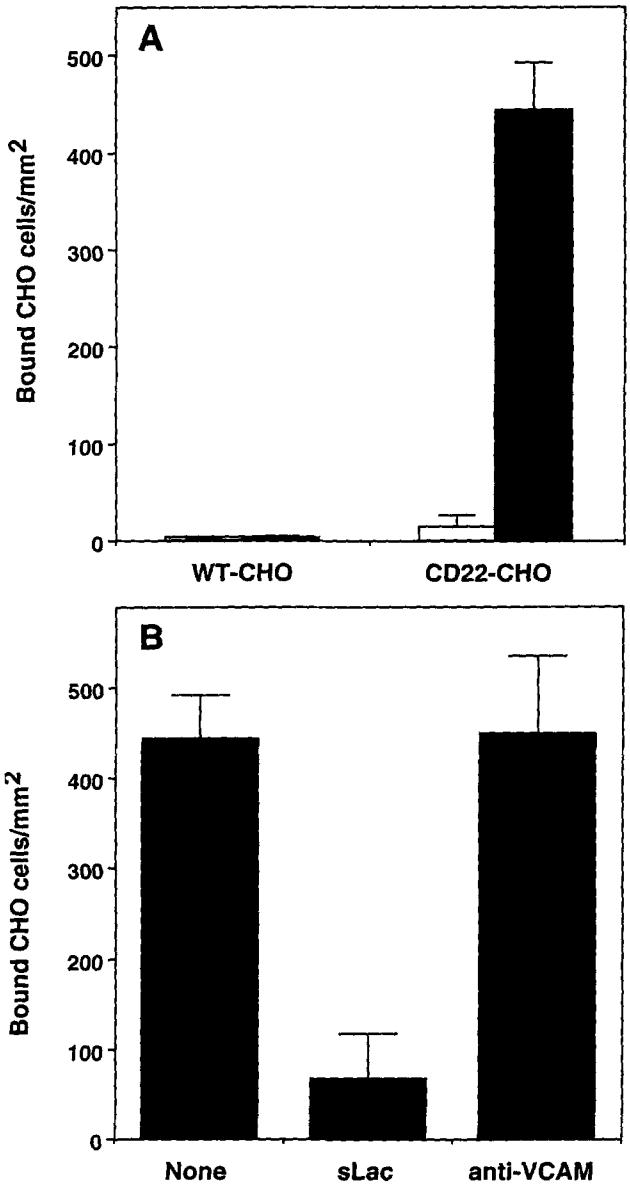

FIG. 4. **Binding of CD22-transfected CHO cells to HEC.** *A*, binding of wild-type (WT-CHO) and CD22-expressing (CD22-CHO) CHO cells to resting (*open bars*) and TNF-α-stimulated (*closed bars*) HEC. Confluent HEC were stimulated with or without 200 units/ml TNF-α for 48 h. EDTA-harvested CHO and CD22-CHO cells were added to HEC monolayers (1×10^5 cells/well) and incubated for 30 min at 4 °C. After washing, the adherent cells were fixed and counted microscopically. *B*, effect of α2–6-sLac on CD22-transfected CHO cell binding to TNF-α-stimulated HEC. TNF-α-stimulated HEC were pretreated with or without anti-VCAM-mAb, and incubated with CD22-transfected CHO cells in the absence or presence of 1.5 mM α2–6-sLac. For *A* and *B*, the data are the mean ± S.E. of triplicates of a representative experiment ($n = 3$). The level of expression of CD22 on these CHO transfectants is indicated in Fig. 5.

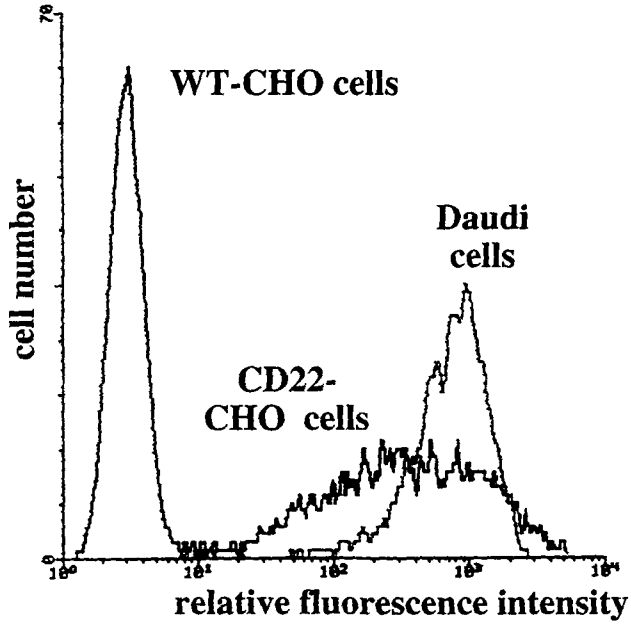

FIG. 5. **Flow cytometry analysis of CD22 on transfected CHO and Daudi cells.** Wild-type and CD22-transfected CHO cells (EDTA-harvested) and Daudi cells were stained with PE-conjugated anti-CD22 mAb BL22 and analyzed as described under "Experimental Procedures." Similar staining patterns were obtained using another anti-CD22 mAb Leu-14.

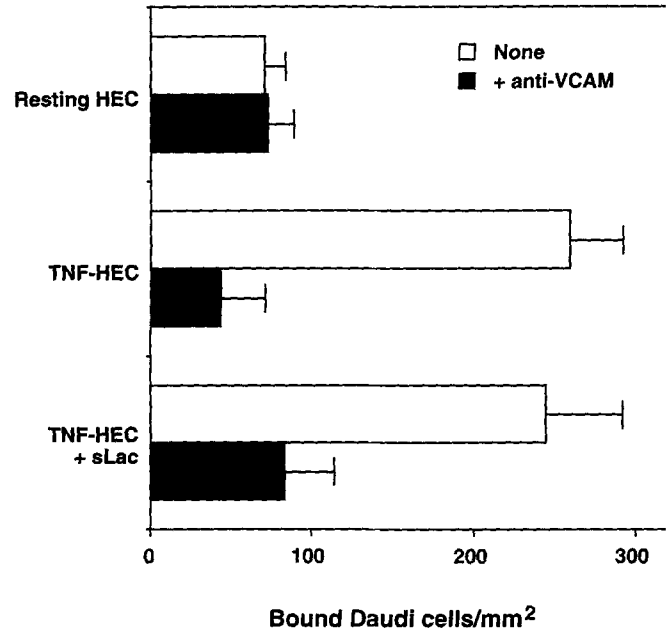

FIG. 6. **Adhesion of Daudi cells to TNF-α-stimulated HEC.** Effects of anti-VCAM-mAb and α2–6-sLac on Daudi cell adhesion to TNF-α-activated HEC. After stimulation with or without 200 units/ml TNF-α for 48 h, HEC were pretreated with or without anti-VCAM-mAb, and incubated with Daudi cells (1×10^5 cells) in the absence or presence of 1.5 mM α2–6-sLac for 30 min at 4 °C. After washing, the adherent cells were fixed and counted microscopically. The data are the mean ± S.E. of triplicates of a representative experiment ($n = 3$).

the integrin $\alpha_1\beta_4$ of the Daudi cells). Similar VCAM-1-dependent adhesion was observed using another B cell lymphoma line (Raji) that also expresses high levels of CD22 (data not shown and Ref. 13). Again, no inhibition was seen with α2–6-sLac (data not shown). Taken together, these results suggest that binding of CD22-bearing B cell lymphomas to cytokine-activated endothelium is primarily mediated by the increased expression of VCAM-1, and not by the lectin function of CD22. Indeed, the cell-surface CD22 of these lymphoma cells might be nonfunctional with regard to its α2–6-Sia binding property.

Direct Probing of CD22 Lectin Function on Cell Surfaces of CD22-transfected CHO Cells and Daudi Cells—Flow cytometry analysis using FITC-labeled SNA, a plant lectin that recognizes α2–6-linked Sia (47), confirmed data from other published studies (29, 30, 32, 33, 48) showing that B cell lymphoma cell lines express high levels of α2–6-linked Sias on their cell surfaces (data not shown). In contrast, wild-type and CD22-transfected CHO cells express hardly any detectable α2–6-

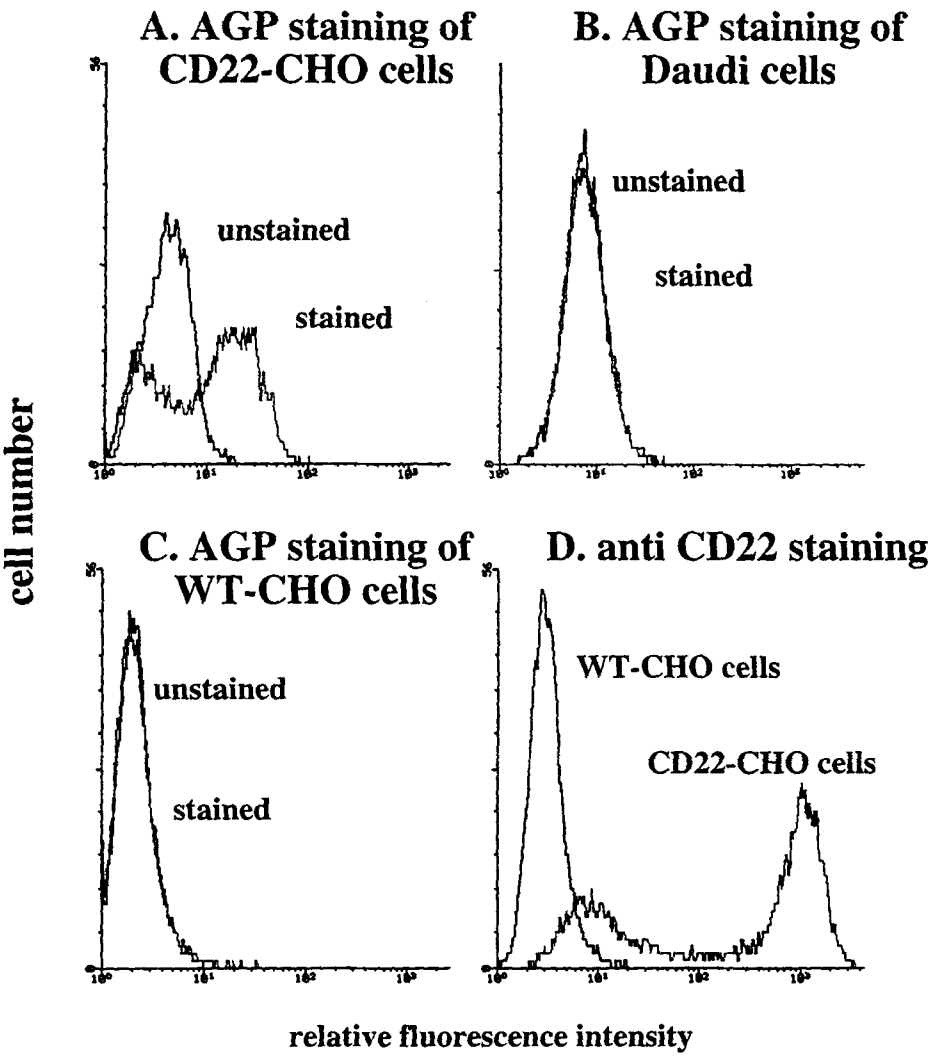

FIG. 7. **Detection of lectin activity of cell-surface expressed CD22.** FITC-tagged AGP was used to stain CD22-CHO cells (*panel A*), Daudi cells (*panel B*), or WT-CHO cells (*panel C*). The expression of cell-surface CD22 on the CD22-CHO cells used here is similar to those of Daudi cells (data not shown), although this particular clone contained a subpopulation that are negative, see *panel D*. This likely explains the incomplete staining seen in *panel A*.

linked Sias (data not shown). These results raised the possibility that the lectin property of CD22 on B cell lymphomas might be rendered nonfunctional by endogenous α2–6-sialylated ligands. To explore this hypothesis, the lectin function of the CD22 molecule was probed directly using FITC-labeled AGP, a fraction of which had previously been demonstrated to exhibit high affinity binding to CD22Rg (42). (Although the accompanying paper (49) shows that IgM is an even better ligand, this protein showed high nonspecific binding to wild-type CHO cells and could not be used as a specific probe.) The FITC-labeled AGP probe was capable of staining CD22-expressing CHO cells (Fig. 7) and not parental CHO cells (data not shown), indicating that it probes the lectin function of CD22 on the cell surface (this particular batch of CD22-CHO has a subpopulation of CD22-negative revertants, explaining the biphasic staining distribution). This probe did not stain Daudi cells (Fig. 7), which have slightly higher levels of cell-surface CD22, nor Raji cells (data not shown). Thus, the lectin activity of CD22 is functionally "masked" on these B lymphoma cell lines. A recent study by others utilizing transient expression of CD22 and ST6N (39) would suggest that this masking is due to endogenous sialylated ligands. To explore this possibility, we tried pretreatment of the lymphoma cells with either sialidase or sodium periodate, which are known to destroy CD22 ligands. These treatments failed to "unmask" the CD22 lectin activity (data not shown). However, since these techniques are only partially effective on cell surfaces, a low level of Sia residues might survive and be sufficient to block the lectin activity of CD22. Alternatively, the critical Sia residues might be selectively protected from these treatments by virtue of their involvement in binding endogenous CD22.

CHO Cells Coexpressing CD22 and ST6N Do Not Bind to Activated HEC—Since attempts to remove or destroy cell-surface Sia residues were not successful in restoring the lectin property of CD22 on the B cell lines, an alternative approach was needed to demonstrate that endogenous Sia residues can regulate the lectin activity of cell-surface CD22. For this purpose, CHO cells stably transfected with ST6N and/or CD22 were isolated. By carefully selecting singly or doubly-transfected clones, lines were obtained with comparable levels of expression of α2–6-Sia structures (SNA staining) and/or CD22 (BL22 staining) (data not shown). As shown in Fig. 8, coexpression of ST6N with CD22 in the CHO cells completely abrogated their binding activity to cytokine-activated HEC. This demonstrates that endogenous α2–6-linked Sia-dependent ligands can suppress the CD22 binding to the sialylated ligands of endothelial cells.

Differential Inhibition by α2–6-sLac of B Lymphoma Cell Binding to CD22-positive or CD22/ST6N-positive CHO Cells—As shown in Fig. 9*A*, CD22-transfected CHO cells bound Daudi B lymphoma cells, whereas WT-CHO cells did not. This interaction was blocked by 1 mM α2–6-sLac but not by α2–3-sLac (data not shown). Thus, as expected, the CD22 molecule expressed on CHO cells can function as a lectin that recognizes

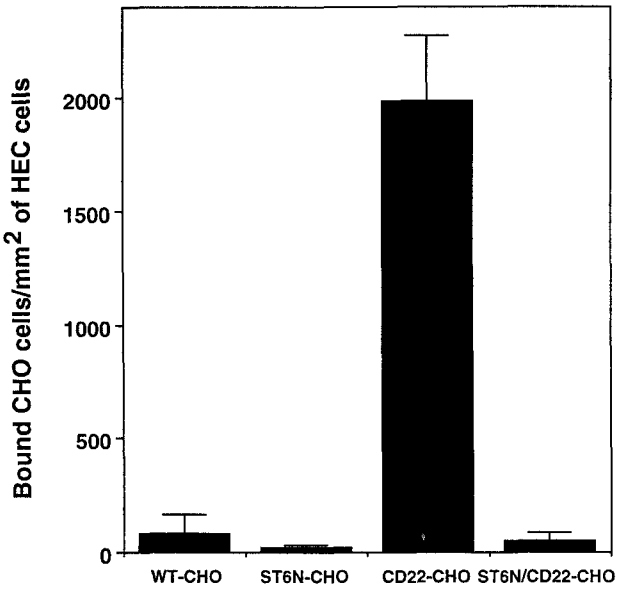

FIG. 8. **Binding of CHO cells transfected with CD22 and/or ST6N to TNF-α-stimulated HEC.** Confluent HEC were stimulated with 200 units/ml TNF-α for 48 h. EDTA-harvested WT-CHO, ST6N-CHO, CD22-CHO, and CD22/ST6N-CHO cells were added to HEC monolayers (4×10^5 cells/well) and incubated for 30 min at 4 °C. After washing, the adherent cells were fixed and counted microscopically. The data are the mean ± S.E. of triplicates of a representative experiment ($n = 3$).

the α2–6-linked Sias on Daudi cells. However, CHO cells coexpressing both CD22 and ST6N (which could not bind to α2–6-linked Sias on activated HEC, see Fig. 8) could still bind Daudi cells (Fig. 9*A*). α2–6-sLac suppressed the adhesion of both types of transfected CHO cells to Daudi cells. Notably, the binding of CD22/ST6N-positive CHO cells was much less sensitive to the inhibition by α2–6-sLac than that of CHO cells transfected with CD22 alone (Fig. 9*B*). This raises the possibility of another CD22-dependent adhesion mechanism not directly dependent on its lectin function. However, a higher concentration (3 mM) of α2–6-sLac was effective in blocking this adhesion, indicating the possibility of an indirect effect involving α2–6-linked Sias.

Cross-binding Studies of CHO Cells Expressing CD22 and/or ST6N—Given the contrasting results with the binding of ST6N/CD22-CHO cells to HEC *versus* Daudi cells, an additional set of cross-binding experiments was performed. Aliquots of the four different CHO sublines (WT-CHO, CD22-positive, ST6N-positive, and CD22/ST6N-positive, selected for comparable levels of expression by flow cytometry) were labeled with [^{3}H]thymidine, lifted from tissue culture by PBS/EDTA (without trypsin), and tested for cross-adherence to the same four sublines presented as intact monolayers. As shown in Fig. 10, only the CD22-expressing cells exhibited adherence above background to either of the ST6N-expressing cells. Cells which coexpressed ST6N and CD22 (CD22/ST6N-CHO monolayer cells, Fig. 10) failed to show increased binding either to themselves or to the ST6N-expressing cells. This confirms that the lectin function of CD22/ST6N-CHO cells is functionally inactive. Thus, their binding to Daudi cells (which are also CD22/ST6N-positive and have functionally inactive CD22) must be due to additional mechanisms specific to the Daudi cells.

DISCUSSION

The sequential steps of leukocyte rolling, activation, adhesion, and extravasation into inflammed tissues involves several receptor-ligand pairs, which are themselves often under control

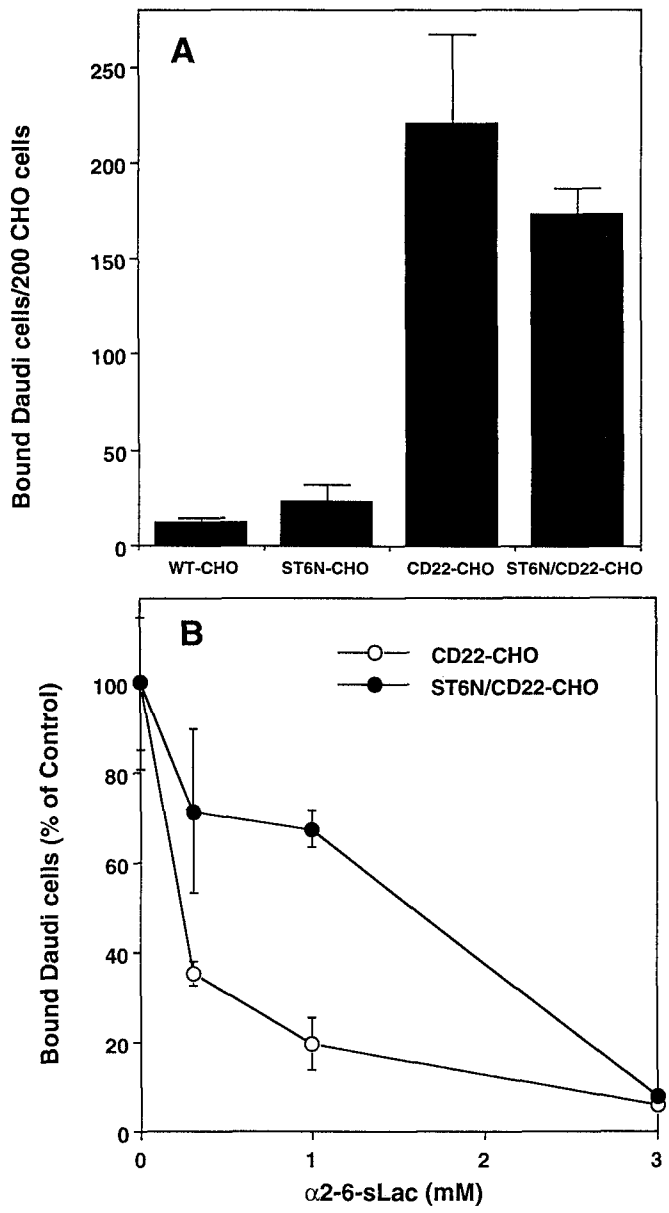

FIG. 9. **Binding of transfected CHO cells to Daudi cells.** *A*, binding of transfected CHO cells to Daudi cells. Daudi cells were added to monolayers of parental or transfected CHO cells, and incubated for 30 min at 4 °C. After washing, the adherent cells were fixed and the total number of bound Daudi cells to 200 CHO cells counted microscopically. The data are the mean ± S.E. of triplicates of a representative experiments ($n = 3$). *B*, effect of α2–6-sLac on binding of Daudi cells to transfected CHO cells. [^{3}H]Thymidine-labeled Daudi cells were incubated with transfected CHO cells in the presence of various concentrations of α2–6-sLac for 30 min at 4 °C. After washing, the bound cells were solubilized and their radioactivity was counted. Results are expressed as percent of total binding obtained in the absence of α2–6-sLac after subtracting the background levels (obtained in the binding of ^{3}H-labeled Daudi cells to CHO cells). The data are the mean ± S.E. of triplicate determinations.

of several different cytokines (37, 50). Cultured endothelial cells such as HEC are frequently employed for studies of both leukocyte adhesion and cytokine regulation. When exposed to TNF-α, IL-1, or LPS, these cells respond with increased levels of expression of VCAM-1, ICAM-1, P-selectin, and E-selectin (37, 50), and an as yet unidentified L-selectin ligand (51, 52). In parallel, stimulation often results in increased levels and/or activity of adhesion molecules on monocytes, lymphocytes, and granulocytes (37, 50). Although IL-4 is recognized as a lymphocyte cytokine, recent evidence indicates that it can also activate

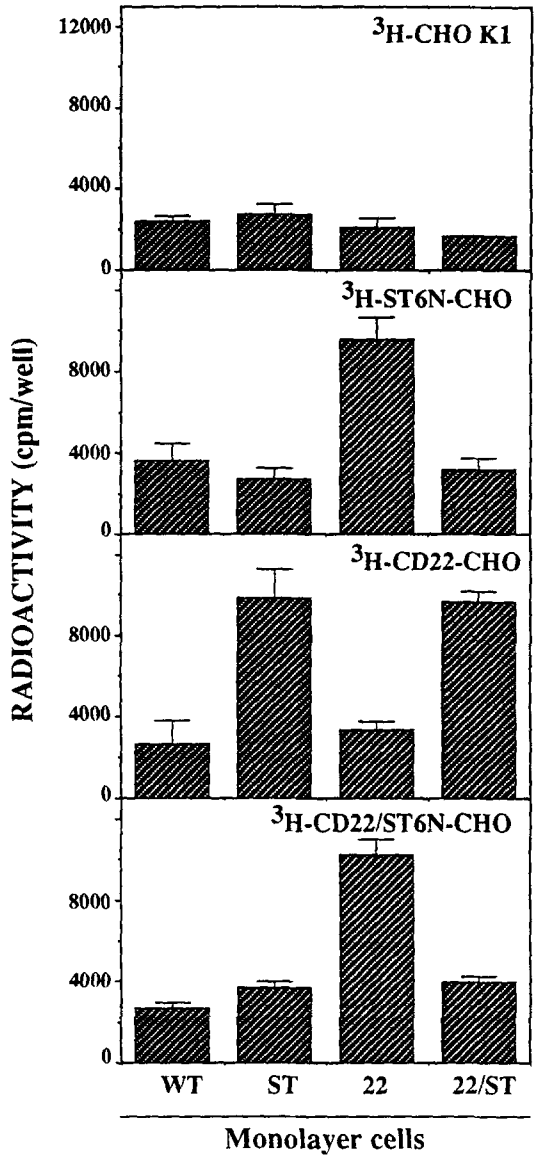

FIG. 10. **Cross-binding patterns between different CHO sublines transfected with CD22 and/or ST6N.** Using the wild-type CHO cells and the three sublines stably transfected to express ST6N (*"ST"*), CD22 (*"22"*), or both (*"22/ST"*), a series of binding assays was performed. The four different cell lines, labeled with [^{3}H]thymidine for 18 h and detached with PBS-EDTA, were added to confluent monolayer cultures of the four different cell lines. After adhesion for 30 min on ice, nonadherent cells were washed away and bound cells quantitated by scintillation counting as described under "Experimental Procedures." Equal numbers of cells of approximately equal cpm (±10%) were added; each *bar* represents the average of triplicate determinations ± S.D.

endothelial cells (45) in a manner significantly different from that by TNF-α, IL-1, or LPS (induction of just VCAM-1 and L-selectin ligand, but not the other adhesion molecules). Moreover, IL-4 stimulation of HEC increases the adhesion of lymphocytes, basophils, eosinophils, and monocytes, but not of neutrophils (45). Thus, the coordinate actions of different cytokines serves to regulate these essential steps in the inflammation pathway. Much of this work has focused on the trafficking of neutrophils or T cells into inflammed tissues, and the migration of T or B lymphocytes into normal lymphoid organs (37, 50).

We recently demonstrated that TNF-α, IL-1, or LPS stimulation of HEC cells results in increased levels of expression of ST6N (both mRNA and enzyme activity) and of total cellular α2–6-linked Sia residues (38). The latter can be detected by increased levels of binding of the lectin SNA, as well as by soluble recombinant CD22Rg, both of which require α2–6-linked Sia residues for recognition. Here, we show that the cytokine IL-4 can also induce such a response, and generalize these results by showing ST6N responses to cytokines in human dermal microvasculature endothelial cells as well.

CD22Rg specificially precipitates a family of glycoproteins from HEC cells, including several in the molecular mass range of 130–150 kDa, and some above 200 kDa. The increased binding of CD22 to activated HEC is not explained by synthesis of new and superior ligands, because the pattern of precipitated glycoproteins remain unchanged. This observation contrasts with our previous result that when glycoproteins of TNF-α stimulated HEC cells are stained with the lectin SNA, only a few new protein bands appear (38). Notably, while VCAM-1 represents a dominant TNF-α-induced SNA-staining glycoprotein (38), it is not prominent in CD22Rg precipitates of metabolically-labeled glycoproteins. Thus, while identity of the proteins precipitated from HEC cells by CD22Rg remain unknown, they seem to represent a subset of the total proteins bearing α2–6-linked Sia residues, and are not primarily the previously known cytokine-inducible adhesion molecules. This fits well with our previous observations that some α2–6-sialylated serum glycoproteins exhibited unexpectedly poor binding affinity to CD22Rg (42), and that only certain α2–6-sialylated glycoproteins from activated lymphocytes or ST6N-expressing COS cells are precipitated by CD22Rg (9). Furthermore, as we show in the accompanying paper (49), IgM and haptoglobin are the dominant CD22-binding proteins in human serum, despite the fact that this is a rich source of many other abundant α2–6-sialylated glycoproteins. The structural basis for the selective recognition of α2–6-sialylated glycoproteins by CD22 is partly explored in the accompanying paper (49).

Since no new CD22Rg-precipitable glycoproteins are identified after TNF-α stimulation, the increase in CD22Rg binding could be due to increased levels of constitutively expressed sialylated glycoproteins and/or to changes in their sialylation. In support of the latter, we observed a doubling of the content of *N*-linked oligosaccharides containing two or more α2–6-linked Sia residues. Direct binding studies presented in the accompanying paper (22) indicate that such bi-α2–6-sialylated biantennary oligosaccharides bind to CD22Rg 30-fold better than do mono-α2–6-sialylated structures, probably owing to the interaction of the two α2–6-Sia binding sites present on the bivalent CD22Rg chimera. The increased level of bi-α2–6-sialylated structures in the activated HEC fits well with the previously reported increase in ST6N (both activity and mRNA) (38). Of course, other factors may also affect the actual content of α2–6-Sia residues (*e.g.* competing glycosyltransferases and oligosaccharide branching), and have not been examined here.

It is difficult to isolate pure populations of truly unactivated mature CD22-positive B lymphocytes from humans, and no cultured cell lines properly recapitulate this phenotype. In particular, rapidly growing CD22-positive B cell lines are, by their very nature, "activated" and coexpress high levels of α2–6-linked Sia (30, 32–34, 53). While the latter phenotype is relevant to the biology of the activated B cell, it is necessary to use transfected cell lines to study the adhesion properties of CD22 in isolation. Others have previously done this using transient expression of CD22 in COS cells (39). While this can give useful results, the rather high and unpredictable levels of cell-surface CD22 generated could potentially produce artifacts. Therefore, we prepared stable transfectants with CHO cells expressing either the full-length human CD22, the human

TABLE I
Summary of cell-cell binding experiments

Relative binding is reported on a scale of − to +++, based upon the data presented in Fig. 4, Figs. 6–10, and other data not shown. In each case, the cells to be bound were in suspension, and the cells they bound to were in monolayers. For purposes of this summary, background binding by the wild-type (WT)-CHO cell line is indicated as negligible (−).

Cells Bound	Binding to					
	Resting HEC	Activated HEC	WT-CHO	CD22-CHO	ST6N-CHO	CD22/ST6N-CHO
WT-CHO	−	−	−	−	−	−
ST6N-CHO	−	−	−	+++[a]	−	−
CD22-CHO	+	+++[a]	−	−	+++[a]	+++[a]
CD22/ST6N-CHO	−	−	−	+++[a]	−	−
Daudi B lymphoma	−	+++[b]	−	+++[a]	−	+++[c]

[a] α2–6-Sia dependent binding.
[b] Anti-VCAM-1 sensitive, not α2–6-sLac sensitive.
[c] α2–6-Sia dependent binding, but requires more α2–6-sLac to achieve inhibition.

ST6N enzyme, or both. To ensure comparability, specific clones were selected that express CD22 at levels (by flow cytometry analysis) similar to those seen in B lymphoma cell lines.

As expected from the results using soluble CD22Rg, CD22-expressing CHO cells bind well to TNF-α-stimulated HEC. However, the improvement in binding seen over the unactivated HEC is much more dramatic than might be expected from the modest (2-fold) increase in bi-α2–6-sialylated oligosaccharides (compare Fig. 3 with Fig. 4). This may be because cell adhesion assays are more dependent upon the absolute density of given receptor-ligand pairs than are enzyme-linked immunosorbent assay analyses using soluble receptors, *i.e.* the "threshold effect" (54). The inhibition of adhesion by α2–6-sLac and the lack of binding of nontransfected wild-type CHO cells to TNF-α-stimulated HEC indicates that other adhesion molecules expressed by CHO cells are not involved. Thus, the increase in ST6N in activated endothelium might be sufficient to exceed the threshold for B cells to bind *in vivo*.

In the normal immune system, early activated B cells are expected to carry both CD22 and CD22 ligands on their cell surfaces (13, 55). Similar coexpression of both CD22 and its ligands is found with B lymphoma cells, such as Daudi and Raji cells (13). Since the B cells of the mantle zone of secondary follicles in lymph nodes are in an activated state, express CD22 (55), and also express α2–6-sialylated structures which are potentially ligands for CD22 (30), it is important to know if CD22 can still mediate cell adhesion under such circumstances. In this regard, the recent report of Braesch-Anderson and Stamenkovic (39) demonstrated that when CD22 is transiently coexpressed with ST6N in COS cells, a loss of binding to sialylated structures results. Our results here with CHO cell lines stably expressing CD22 and/or ST6N have confirmed and extended this observation. In addition, direct probing of the lectin function of CD22 on cell surface by AGP staining indicates a loss of function in the Daudi B lymphoma cells. Thus, ST6N expression can regulate CD22-mediated adhesion both negatively (if expressed on cells expressing CD22) and positively (if expressed on potential target cells).

A summary of most of the cell binding results from this paper is presented in Table I. In general, the data are internally consistent, with ST6N expression correlating with binding by CD22-expressing cells, and doubly positive cells showing lack of lectin-mediated binding. The only unexpected results were obtained with Daudi B lymphoma cells, which express both CD22 and ST6N, and do not demonstrate CD22 function on the surface by direct probing. As expected from all other results, they do not bind to HEC in a sialic acid-dependent manner. Despite this, they bind to CHO cells coexpressing CD22 and ST6N, which are themselves deficient in the lectin activity (see Table I). This interaction is dependent upon the presence of CD22 on the CHO cells (there is no binding to CHO cells expressing ST6N only). While this interaction was inhibited by high concentrations of α2–6-sLac, it was not affected by the coexpression of ST6N with CD22 on the CHO cells. Three possible explanations of these observations are suggested. First, Daudi cells may express such high levels of α2–6-sialylation that they can efficiently compete with the endogenous α2–6-sialylated structures expressed on the ST6N/CD22-CHO cells. Second, Daudi cells may express a lymphocyte-restricted sialoglycoprotein ligand capable of superior binding to very small numbers of active CD22 molecules on the CD22/ST6N-CHO cells that are not masked by endogenous ligands. Additionally, Daudi cells are known to express other adhesion molecules not found on CHO cells (45, 56), which could be contributing. Finally, the previously suggested homotypic interaction between CD22-positive cells (18) might depend upon endogenous α2–6-sialylation (causing a conformational change in the CD22 molecules), which can presumably be blocked by high concentrations of exogenously added α2–6-sLac. The available data provides partial support for each of the hypotheses. First, a higher percentage of *N*-linked oligosaccharide purified from Daudi cells contain two and three α2–6-linked Sia residues than *N*-linked oligosaccharides from ST6N-expressing CHO cells (Fig. 3).[2] Second, Daudi cells express CD45, which is known to be a high affinity ligand for CD22, while CHO cells do not. Finally, Daudi cells do show significant clumping among themselves in undisturbed cultures. These observations point to the complexities of cell adhesion processes, and the need for further studies. Regardless, they clearly establish that cell activation status can regulate CD22-dependent adhesion events by affecting expression of both CD22 and ST6N.

The "autoinactivation" of CD22 lectin activity by endogenous oligosaccharide ligands reported here and elsewhere (39) is not without precedent in vertebrate lectin biology. In early studies of the hepatocyte asialoglycoprotein receptor, it was noted that sialidase treatment of hepatocytes causes loss of receptor activity because of binding to newly generated endogenous ligands (57); activity could then be restored by resialylation (58). It was subsequently suggested that this might be a natural mechanism to regulate the activity of this receptor (59). It is noteworthy that there are other situations where this could potentially occur. For example, neutrophils constitutively express L-selectin as well as large amounts of cell surface sialyl-Lewis^x, which is considered to be a low affinity ligand for this receptor (60). The possibility that this selectin is partly occupied by these endogenous ligands has not been explored.

Finally, the relevance of the interaction of CD22-positive cells with activated endothelium needs to be explored. Resting B cells possess CD22 on the cell surface without high level expression of ST6N. During inflammatory processes, these cells could bind to activated vascular endothelium via recognition of CD22 ligands, perhaps to obtain partially processed antigen from tissues, and/or to traffic into the inflammed tissues sites. While these two processes are not part of current dogma concerning B cell trafficking and function, they are now worthy of consideration. However, as discussed in the accompanying paper (49), such interactions would have to take place in the presence of blood plasma which contains many sialylated glycoproteins, some of which are potent inhibitors of CD22. It appears likely that other pairs of adhesion molecules (*e.g.* L-

[2] L. Powell, unpublished data.

selectin and its ligand on activated endothelium) would have to contribute substantially toward an initial binding event.

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