THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 13, Issue of March 31, pp. 7543–7550, 1995
Printed in U.S.A.

Binding of Human Plasma Sialoglycoproteins by the B Cell-specific Lectin CD22

SELECTIVE RECOGNITION OF IMMUNOGLOBULIN M AND HAPTOGLOBIN*

(Received for publication, November 30, 1994, and in revised form, January 13, 1995)

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CD22 is a cell-surface receptor of resting mature B cells that recognizes sialic acid (Sia) in the natural structure Siaα2–6Galβ1–4GlcNAc (Powell, L. D., Jain, R. K., Matta, K. L., Sabesan, S., and Varki, A. (1995) *J. Biol. Chem.* 270, 7523–7532). Human umbilical vein endothelial cells (HEC) treated with inflammatory cytokines such as tumor necrosis factor-α (TNF-α) display increases in cell-surface CD22 ligands, caused by increased expression of the enzyme β-galactoside α2,6-sialyltransferase (Hanasaki, K., Varki, A., Stamenkovic, I., and Bevilacqua, M. P. (1994) *J. Biol. Chem.* 269, 10637–10643; Hanasaki, K., Varki, A., and Powell, L. D. (1995) *J. Biol Chem.* 270, 7533–7542). Thus, CD22 could direct potential interactions between mature B cells and endothelial cells during inflammatory states. However, this would have to occur in the presence of blood plasma, which contains many sialoglycoproteins known to carry α2–6-linked sialic acids. We show here that human plasma can indeed inhibit Sia-dependent binding of a recombinant soluble chimeric form of human CD22 (CD22Rg) to TNF-α activated HEC. Affinity adsorption of individual human plasma samples with immobilized CD22Rg showed that, of the numerous α2–6-sialic acid containing glycoproteins in plasma, only three polypeptides with apparent molecular mass (under reducing conditions) of 74, 44, and 25 kDa bound, and were specifically eluted with α2–6-sialyllactose. NH_2-terminal amino acid sequencing of these high affinity CD22 ligands revealed that they are subunits of immunoglobulin M (IgM) and haptoglobin. Purified human IgM from pooled human plasma can be quantitatively bound by CD22Rg, and binding is blocked by α2–6-sialyllactose, but not by α2–3-sialyllactose. Pretreatment by sialidase or by mild periodate oxidation of sialic acid side chains abolishes these interactions. IgM at physiological concentrations also inhibits CD22Rg binding to TNF-α-activated HEC in a manner dependent not only upon its sialylation but also requiring its intact multimeric structure. These data show that CD22 is capable of highly selective recognition of certain multimeric plasma sialoglycoproteins that carry α2–6-linked sialic acids. Notably, the two proteins that are selectively recognized are known to be involved in immune and inflammatory responses. Haptoglobin synthesis by the liver is markedly increased during the "acute phase response" to systemic inflammation, while IgM is the major product resulting from activation of resting CD22-positive B cells.

CD22 is a cell-surface phosphoglycoprotein found on the majority of resting mature B cells, and appears to be involved in antigen-induced cell activation (1, 2) and in cell adhesion, mediating interactions with activated blood cells, accessory cells, and endothelial cells (3–7) (see also the accompanying papers (8, 9)). Soluble chimeric forms of CD22 (CD22Rg)[1], containing the three amino-terminal Ig-like domains of human CD22β fused to the COOH-terminal Fc domains of human IgG or mouse IgG (10, 11) have been previously used to identify sialoglycoprotein ligands on activated T and B cells, which include, among others, CD45, the leukocyte-specific receptor-linked phosphotyrosine-phosphatase (5, 10, 12). These interactions involve recognition of the sialic acid (Sia) containing structural motif Siaα2–6Galβ1–4GlcNAcβ1- (8, 11, 13, 14). This sequence is known to occur in varying copy numbers on the *N*-linked oligosaccharides of some cell-surface glycoproteins (15). The Sia residues of these glycoprotein ligands are essential for binding to CD22, since the interaction is blocked by their pretreatment with sialidase or by mild periodate oxidation under conditions which are specific for truncation of the exocyclic side chain of sialic acid (6, 10, 11).

Recent studies have demonstrated some potential regulatory mechanisms that could affect CD22-dependent adhesion events *in vivo*. Activation of T lymphocytes causes enhanced expression of CD22 ligands (10), apparently via increased expression of β-galactoside α2,6-sialyltransferase (16), the enzyme which transfers terminal α2–6-linked Sia to Galβ1–4Glc(NAc) (17, 18). This favors the notion that CD22 functions as a co-receptor in T-B cell interactions (5, 12). However, a similar enhancement in expression of β-galactoside α2,6-sialyltransferase and of CD22 ligands also occurs during activation of resting B lymphocytes (10, 19, 20). Indeed, coexpression studies have shown that the lectin function of CD22 can be abrogated by sialylation of CD22 itself with β-galactoside α2,6-sialyltransferase (9, 21). Thus, it has been suggested that CD22-ligand interactions can be positively or negatively regulated by the expression of this sialyltransferase (9, 21). In addition, some

* This work was supported by United States Public Health Service Grant RO1GM32373 (to A. V.) and Clinical Investigator Award KO1 CA01649 (to L. D. P.), and by American Cancer Society Institutional Grant ACS-IRG93W (to L. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CD22Rg, recombinant soluble chimera containing Ig domains 1–3 of human CD22β fused to the COOH-terminal Fc domains of human IgG; TNF-α, tumor necrosis factor-α; CD22 mRg, similar construct with Fc domains of mouse IgG; HEC, human umbilical vein endothelial cells; PAS, protein A-Sepharose; Sia, sialic acid; sLac, sialyllactose; TBS, Tris-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Ab, antibody.

CD22 ligands in cells of lymphoid tissues appear to be "masked" by 9-*O*-acetylation of α2–6-linked sialic acids, a naturally occurring modification which markedly reduces binding to CD22 (14). Finally, we recently found that treatment of human umbilical vein endothelial cells (HEC) with inflammatory cytokines such as tumor necrosis factor-α (TNF-α), causes increased expression of β-galactoside α2,6-sialyltransferase, and enhanced expression of CD22 ligands (detected by CD22Rg binding) (7). As shown in the preceding paper (9), this is accompanied by enhanced binding of Chinese hamster ovary cells expressing transfected human CD22. Thus, we considered the possibility that CD22-expressing B lymphocytes might bind to activated endothelium during inflammatory conditions. This could theoretically mediate extravasation of B cells into tissues, antigen transfer to B cells, and/or some other unknown biological interactions. However, this intercellular recognition process would have to occur in the presence of blood plasma, a rich source of sialoglycoproteins, many of which carry α2–6-linked sialic acids (22). Indeed, we found that human plasma is capable of inhibiting this interaction. In pursuing this finding, we have identified the major high-affinity plasma ligands for CD22, which are dependent upon α2–6-linked sialic acids for recognition.

EXPERIMENTAL PROCEDURES

Materials—Development and characterization of a soluble CD22Rg containing the three amino-terminal Ig-like domains of human CD22β fused to the C-terminal Fc domains of human IgG (CD22Rg) or mouse IgG (CD22 mRg), CD8Rg or L-selectin-Rg has been described previously (10, 11). The following reagents and chemicals were obtained from commercial sources indicated: α2–3- and α2–6-sialyllactose (sLac), Oxford GlycoSystems, Abington, United Kingdom; BSA, 1,2-diamino-4,5-methylenedioxybenzene, iodoacetamide, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, *o*-phenylenediamine, IgG, porcine heparin, and $NaBH_4$, Sigma; *Arthrobacter ureafaciens* sialidase, 2,3-dihydro-2-deoxy-*N*-acetylneuraminic acid, IgM, haptoglobin, and peroxidase-conjugated streptavidin, Calbiochem, San Diego; protein A, Pierce; biotin-conjugated goat anti-human μ-chain specific antibody (Ab) and biotin-conjugated goat anti-human IgG Fc chain specific Ab, Jackson ImmunoResearch; streptavidin-conjugated alkaline phosphatase and proteinase K, Life Technologies, Inc.; protein A-Sepharose (PAS), Pharmacia Biotech Inc.; Immobilon-P membrane, Millipore; Centricon-10 concentrator, Amersham; diisopropyl fluorophosphate, Aldrich; peroxidase-conjugated goat anti-mouse IgG Ab and dithiothreitol, Bio-Rad; sodium periodate, Fisher. Human recombinant TNF-α was a gift from Biogen Corp., Cambridge, MA. Protein concentrations were determined using the bicinchonic acid protein assay reagent kit (Pierce) with BSA as a standard.

Plasma Samples—Since CD22 interactions do not require divalent cations (6, 11), we studied CD22 ligands in whole plasma (anticoagulated in EDTA) rather than in serum, whose protein content is known to be altered during clotting. Blood was collected from healthy human volunteers, and mixed promptly into EDTA (pH 7.35, final concentration 10 mM). After centrifugation at 2,500 rpm for 25 min, the supernatant was collected as plasma.

Sialic Acid Content of Human Samples—Total protein-bound Sia concentrations in samples of human serum or plasma were determined by the 2-thiobarbituric assay after mild acid hydrolysis (23). The relative content of α2–6-linked and α2–3-linked Sia residues was determined by the release of Sia using either Newcastle Disease Virus sialidase (α2–3 specific) or *A. ureafaciens* sialidase (cleaves α2–6-linked and α2–3-linked Sia residues). For the enzyme digestions, 10-μl samples were incubated with 4 milliunits of either enzyme in 50 mM sodium acetate (pH 5.5) for 2 h at 37 °C, in the presence of 0.01% butylated hydroxytoluene to limit lipid peroxidation. Released Sia was determined by the TBA assay without acid hydrolysis (23).

Endothelial Cell Culture and Stimulation—Primary cultures of HEC from Clonetics Corp. (San Diego, CA) were grown in 96-well plates coated with 0.1% gelatin using Medium 199 with 20% fetal calf serum, 50 μg/ml endothelial cell growth supplement, and 100 μg/ml porcine heparin, and subcultured using trypsin/versene. Confluent HEC (passages 2–4) were activated by incubation at 37 °C for 48 h with the above medium containing 200 units/ml TNF-α.

Assay for CD22Rg Binding to HEC—After washing with Medium 199, HEC were incubated with 2.5 μg/ml CD22 mRg in Medium 199, 1% BSA at 4 °C for 2 h, in the absence or presence of α2–6-sLac or α2–3-sLac, plasma, IgM, haptoglobin, or IgG. 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 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 Adsorption of High-affinity Plasma Ligands for CD22—Plasma (1 ml) was diluted with 1 ml of TBSE buffer (50 mM Tris/HCl, pH 7.3, 150 mM NaCl, and 2 mM EDTA) and applied to a 2-ml PAS column pre-equilibrated with TBSE buffer. The pass-through fraction was collected, which was then incubated with CD22Rg (20 μg) coupled to PAS (20 μl) or CD8Rg-PAS or PAS alone for 16 h at 4 °C. After centrifugation, the supernatants were collected as the unbound fraction. The beads were washed 6 times with TBSE and incubated with buffer alone, 1 mM α2–3-sLac or 1 mM α2–6-sLac for 2 h at 37 °C. The supernatant was collected after centrifugation. Plasma samples or the eluted samples were boiled with Laemmli sample buffer containing 5% 2-mercaptoethanol, and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gel, which was fixed and stained with Coomassie Brilliant Blue.

Amino Acid Sequence Analysis of High-affinity Plasma Ligands for CD22—The α2–6-sLac-eluted fraction collected after incubation of plasma with CD22Rg-PAS as described above was concentrated using a Centricon-10. After SDS-PAGE, electroblotting of proteins to an Immobilon-P membrane was performed under a constant voltage (50 V) for 1 h at 4 °C, as described previously (7). After staining with Coomassie Brilliant Blue, the α2–6-sLac-eluted protein bands were excised, and NH_2-terminal amino acid sequencing of each was performed by the UCSD Biology Department Protein Sequencing Lab. For Western blotting, the Immobilon-P membrane was incubated overnight at 4 °C in Tris-buffered saline (TBS; 10 mM Tris/HCl, pH 8.0, and 150 mM NaCl) containing 4% skim milk. The membrane was incubated for 2 h with biotin-labeled goat anti-human μ-chain specific Ab or biotin-labeled goat anti-human IgG Fc-chain specific Ab in 1% BSA/TBS. It was washed three times with TBS, 0.05% Tween 20, incubated for 1 h with streptavidin-conjugated alkaline phosphatase, and then washed three times with TBS, 0.05% Tween 20. The membrane was then incubated with 0.33 mg/ml nitro blue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$.

Binding Assay of α2–6-sLac-eluted Plasma IgM to CD22Rg—The 96-well plates were coated with protein A (0.5 μg) in phosphate-buffered saline (PBS, pH 9.0) at 4 °C overnight, blocked with 1% BSA/PBS (pH 7.0), and then incubated with 1.3 μg/ml CD22 mRg for 2 h at room temperature. After washing with PBS (pH 7.0), the plates were incubated with the plasma proteins originally eluted by α2–6-sLac from CD22Rg-PAS (100 ng; after dialysis against PBS) in the absence or presence of α2–6- or α2–3-sLac (1 mM) overnight. The plates were washed with PBS and incubated with biotin-labeled goat anti-human μ-chain specific Ab. After washing with PBS containing 0.05% Tween 20, the plates were incubated with peroxidase-conjugated streptavidin (1/1,000 dilution) for 1 h and assayed as described above. Samples with truncated sialic acid were prepared by incubating with 2 mM sodium periodate in ice-cold PBS (pH 7.0) for 30 min followed by addition of 20 mM glycerol in PBS. For sialidase treatment, the samples were incubated with 0.2 units/ml *A. ureafaciens* sialidase in 100 mM sodium acetate buffer (pH 5.5) for 2 h at 37 °C, and the reaction was stopped by adding 5 mM sialidase inhibitor (2,3-dihydro-2-deoxy-*N*-acetylneuraminic acid). Sham-treated samples were obtained by incubating with sialidase in the presence of the inhibitor.

Binding Assays of CD22Rg to Purified IgM—Purified IgM from pooled human plasma (1 mg) was applied to a 2-ml PAS column, and the unbound IgM collected in the pass-through fraction. After washing columns with TBSE buffer, bound IgM was eluted with citrate-phosphate buffer (pH 3.0), immediately neutralized with Tris/HCl (pH 8.0), and dialyzed against PBS (pH 7.0). The PAS-unbound IgM (40 μl) was then applied to the CD22Rg-PAS column (1 mg, 0.15 × 10 cm) at 4 °C. After washing with TBSE buffer at room temperature, the bound IgM was eluted with 1 mM α2–6-sLac. As monitored by SDS-PAGE with silver staining, all of the IgM that passed through the PAS column bound to CD22Rg-PAS and was eluted by α2–6-sLac. After dialysis against PBS, all three IgM preparations (pooled IgM, PAS-bound IgM, and CD22Rg-PAS-bound IgM) were coated in 96-well plates (400 ng

each). After blocking with 3% BSA/PBS, the plates were incubated with 8 μg/ml CD22 mRg in the absence or presence of α2–6-sLac or α2–3-sLac (1 mM), and assayed as described above.

Mild Periodate Oxidation of IgM—Purified IgM from pooled human plasma (250 μg) was treated with 2 mM sodium periodate in ice-cold PBS (pH 7.0) for 30 min, followed by incubation with 20 mM $NaBH_4$ for 30 min at room temperature. Sham-treated IgM was prepared by incubating with premixed $NaBH_4$ and sodium periodate. These treatments did not affect the pentameric structure of IgM, as detected by SDS-PAGE without reduction. After extensive dialysis against PBS (pH 6.0), these IgM preparations were concentrated and washed into PBS (pH 7.0) using a Centricon-10 centrifugation filter.

Proteinase K Digestion of IgM—Purified IgM from pooled human plasma (250 μg) was treated with 10 μg of proteinase K in 50 mM Tris/HCl (pH 8.0) and 150 mM NaCl for 4 h at 50 °C, followed by addition of diisopropyl fluorophosphate (final 1 mM) to inactivate the protease. By SDS-PAGE, IgM was completely degraded into 53–59-kDa fragments (nonreducing conditions), and heavy chains were converted to 35-kDa fragments (reducing conditions). Sham-treated IgM was prepared by similar incubation at 50 °C followed by the addition of diisopropyl fluorophosphate-pretreated proteinase K.

Reduction and Alkylation of IgM—Purified IgM from pooled human plasma (250 μg) was treated with 10 mM dithiothreitol in 50 mM Tris/HCl (pH 8.0) and 150 mM NaCl for 2 h at room temperature, followed by incubation with 100 mM iodoacetamide for 2 h at room temperature. Sham-treated IgM was prepared by incubating with iodoacetamide alone. After extensive dialysis with PBS (pH 7.0), these preparations were concentrated. Analysis by SDS-PAGE under nonreducing conditions showed that the dithiothreitol-treated IgM was irreversibly reduced into heavy and light chains (data not shown).

Assay of Sialic Acid Content in Plasma and IgM—The content of bound Sia in plasma proteins or purified IgM was determined by HPLC analysis as described previously (7). Briefly, sialic acids were released from plasma (2 μl) or purified IgM (20 μg) with 2 M acetic acid for 3 h at 80 °C. The released sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene and analyzed by reverse-phase high performance liquid chromatography using a TSK-gel ODS-120T column (TosoHaas). Although this system can detect most major forms of substituted sialic acids, the only major sialic acid in plasma and IgM was *N*-acetylneuraminic acid. Therefore, quantitation of the released sialic acid was done with known standards of derivatized *N*-acetylneuraminic acid.

RESULTS

Human Plasma Contains Inhibitory Factors That Block CD22Rg Binding to TNF-α-activated HEC—We previously reported that treatment of HEC with inflammatory cytokines such as TNF-α, causes increased expression of CD22 ligands, detected by CD22Rg binding (7) (see also the accompanying paper (9)). This induction of CD22Rg ligands by TNF-α is time-dependent, saturable at 48–72 h, and shows dose dependence, with a half-maximal effect at 2 units/ml TNF-α (data not shown). CD22Rg binding to both unactivated and activated HEC is blocked by α2–6-linked sialyllactose (Siaα2–6Galβ1–4Glc, α2–6sLac) (Fig. 1*A*) at an IC_{50} (concentration inhibiting binding by 50%) of 80 μM (Fig. 1*B*). Binding is not inhibited by the isomer Siaα2–3Galβ1–4Glc (α2–3sLac), confirming the specific role of α2–6-linked sialic acids in binding. We therefore considered the possibility that circulating CD22-positive B cells might recognize activated endothelial cells in sites of inflammation via CD22-ligand interactions. However, this would have to occur in the milieu of blood plasma, which contains a large number of sialylated glycoproteins, many of which carry α2–6-linked Sia residues (15, 22) that could be potential inhibitors. In fact, as shown in Fig. 2, human plasma samples can block CD22Rg binding to TNF-α-activated HEC with an IC_{50} of 3.7 ± 0.4 volume %. Since the protein-bound sialic acid content of plasma samples from four different individuals was measured at 1.42 ± 0.15 mM, this IC_{50} corresponds to an effective sialic acid concentration of 53 ± 6 μM. Analysis by sialidase release showed that the great majority of these Sia residues (80–90%) are α2–6-linked (data not shown). Thus, the actual concentration of protein-bound α2–6-linked Sia in human plasma is at least 1 mM. This finding may also explain the recent report by Engel *et al.* (6) of unidentified substances in serum and ascites fluid that inhibit CD22-mediated adhesion events.

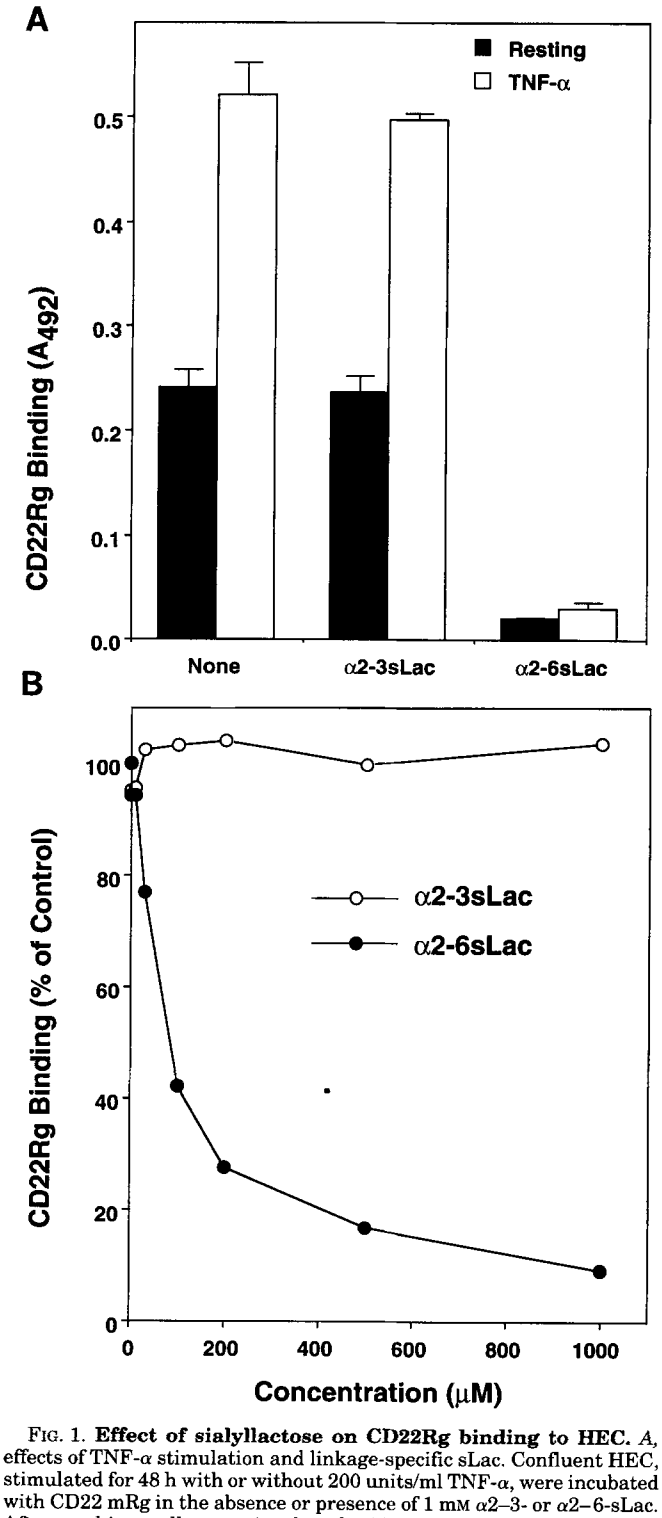

FIG. 1. **Effect of sialyllactose on CD22Rg binding to HEC.** *A*, effects of TNF-α stimulation and linkage-specific sLac. Confluent HEC, stimulated for 48 h with or without 200 units/ml TNF-α, were incubated with CD22 mRg in the absence or presence of 1 mM α2–3- or α2–6-sLac. After washing, cells were 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 triplicates from a representative experiment (n = 4). *B*, effects of sLac concentration. TNF-α-activated HEC were incubated with CD22 mRg in the presence of various concentrations of sLac, and the binding detected as above. The data are the means of duplicates from a representative experiment (n = 3).

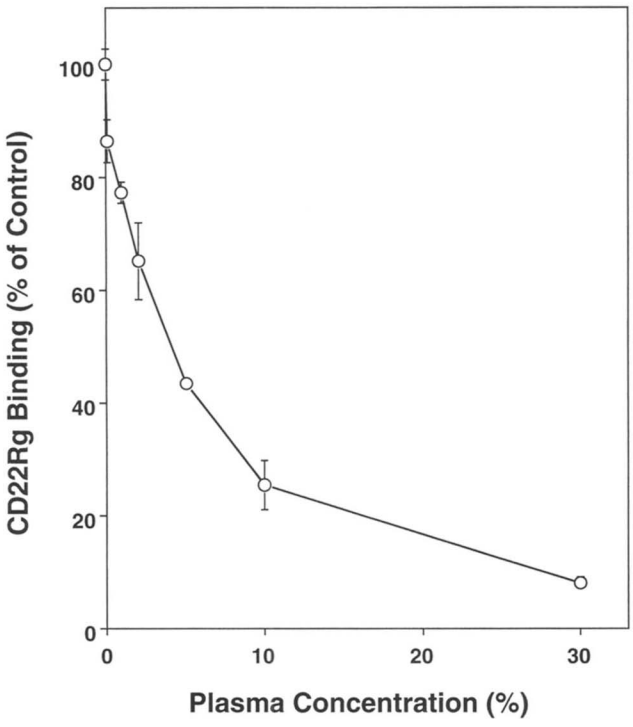

FIG. 2. **Effects of human plasma on CD22Rg binding to TNF-α-activated HEC.** TNF-α-activated HEC were incubated with CD22 mRg in the presence of various concentrations of human plasma and assayed as described in the legend to Fig. 1. Plasma samples from four different individuals were studied. The data shown are the mean ± S.D. of duplicates with each plasma sample.

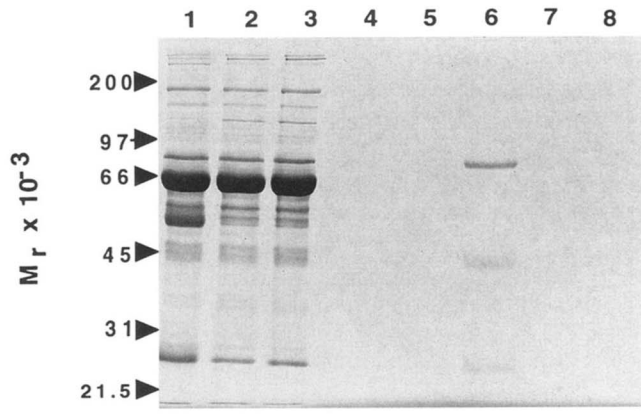

FIG. 3. **Identification of CD22-binding proteins in plasma by affinity adsorption.** Plasma (*lane 1*) was applied to a PAS column. The pass-through fraction was collected (*lane 2*), which was then incubated with CD22Rg-coupled to PAS (*lanes 4–6*), CD8Rg-PAS (*lane 7*), or PAS alone (*lane 8*). After centrifugation, the supernatants were collected as the unbound fraction (*lane 3* shows one example). The beads were washed with TBSE and incubated with buffer alone (*lane 4*), 1 mM α2–3-sLac (*lane 5*), or 1 mM α2–6-sLac (*lanes 6–8*) at 37 °C. The supernatant was collected after centrifugation. Plasma samples (*lanes 1–3*, originating from 0.2 μl of plasma) or the eluted samples (*lanes 4–8*, originating from 20 μl of plasma) were analyzed by SDS-PAGE under reducing conditions.

Identification of High-affinity Plasma Ligands for CD22—Plasma proteins carry sialic acids in a variety of linkages and in varying numbers, and many are known to have multiple copies of the Siaα2–6Galβ1–4GlcNAc sequence recognized by CD22 (15, 22). To identify the high-affinity ligands for CD22 in plasma, we performed an affinity adsorption assay using CD22Rg coupled to PAS. Samples were first precleared by passage through a PAS column which bound mainly the expected 50 kDa (heavy) and 25 kDa (light) chains of IgG (Fig. 3, *lanes 1* and *2*). PAS-unbound proteins were then subjected to affinity adsorption with CD22Rg-PAS. The protocol involves extensive washing steps after the initial binding, ensuring that only high-affinity ligands remain bound. Incubating the washed beads with α2–6-sLac gave elution of proteins with apparent molecular masses of 74, 44, and 25 kDa in reducing SDS-PAGE. No proteins were eluted by incubation with α2–3-sLac, nor by α2–6-sLac when CD8Rg-PAS or PAS alone were used in place of CD22Rg-PAS (Fig. 3). No additional proteins were eluted by boiling the beads in SDS after α2–6-sLac elution (data not shown). Thus, the α2–6-sLac-eluted proteins are specifically bound by CD22, probably via their α2–6-linked sialic acid residues. Similar results were obtained with plasma from three other individuals. In nonreducing conditions, all of the proteins migrated at very high M_r positions which we could not accurately estimate, indicating that they are part of multimeric complexes (data not shown). Treatment with peptide *N*-glycosidase F which releases *N*-linked oligosaccharides, gave shifts in molecular masses of two polypeptides (74 to 65 kDa and 44 to 33 kDa), whereas the 25-kDa band showed little, if any, change (data not shown). The three polypeptides were electroblotted onto Immobilon-P membranes after SDS-PAGE, and subjected to NH_2-terminal amino acid sequence analysis. Computer analyses (GenBank, BLAST, NCBI) of the sequences revealed that the 74-kDa polypeptide (E-V-Q-L-V-E-S-G-V/G-D-L/V-V-Q) belongs to the human Ig heavy chain family and the 25-kDa polypeptide (D/E-I-V/Q-L/M-T-Q-S-P) to the human light chain group. The 74-kDa polypeptide can be stained in Western blotting by anti-human μ-chain specific Ab, but not by anti-human IgG-Ab (data not shown). This, together with the high molecular weight nature of the unreduced complex, confirms the identification of IgM. The NH_2-terminal sequence of 44-kDa protein (I-L-G-G-H-R-L-D) matched precisely with the β-subunit of haptoglobin, an acute phase reactant protein (24–26). To confirm that the initially detected interactions were specific, the α2–6-sLac-eluted samples were dialyzed free of sLac, and studied for rebinding to CD22Rg, which was captured on a microtiter plate by protein A. Binding could be detected using anti-human μ-chain specific Ab, and was completely suppressed by co-incubation with α2–6-sLac (IC_{50} = 40 μM), by pretreatment of the proteins with mild periodate (which selectively truncates the side chains of sialic acids (27–30), or by sialidase (Fig. 4). No binding was observed with the controls, protein A-captured CD8Rg, or L-selectin-Rg.

Effects of Various Treatments on the Interaction of Purified IgM with CD22—Since we used CD22Rg-PAS for the affinity adsorption, it was necessary to first preclear with PAS alone. However, it is known that certain subsets of IgM can bind to PAS via a V-region specific interaction (31). Furthermore, the samples examined were from different individuals, who may express varying ratios of different μ-heavy chain types. To explore whether all IgM subsets can bind to CD22 in a sialic acid-dependent manner, we used commercially available IgM purified from pooled human plasma. About 35% of this IgM bound to the PAS pre-column, and all of the material that passed through this column bound to CD22Rg-PAS, and was eluted by α2–6-sLac (data not shown). However, in plate-binding assays (Fig. 5), CD22Rg reacted equally well with total IgM preparations as with the PAS-bound/citrate eluted or the CD22Rg-PAS-bound/α2–6-sLac-eluted IgM fractions (CD8Rg and L-selectin-Rg controls did not bind to any of these IgM preparations). Similar α2–6-sLac-inhibitable binding of CD22Rg was detected when plates were coated with human haptoglobin, but not with human IgG (1 μg each, data not shown).

As shown in Fig. 6, pooled human IgM blocked CD22Rg

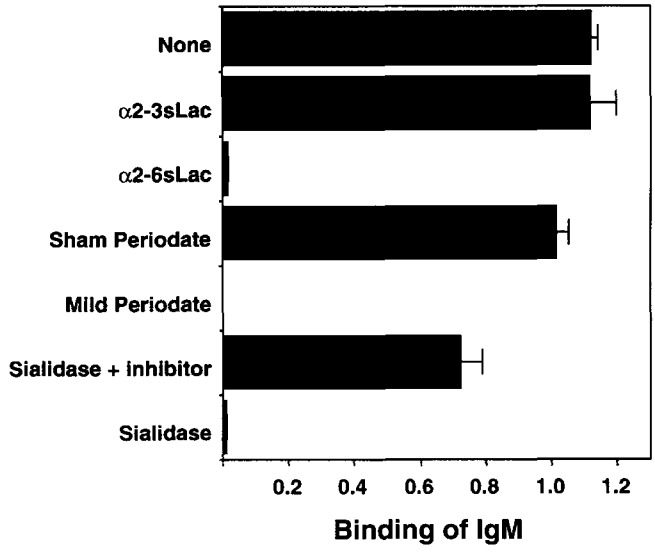

FIG. 4. **Rebinding of α2–6-sLac-eluted plasma IgM to CD22Rg and effects of sialidase or periodate.** Plasma proteins eluted by α2–6-sLac from CD22Rg-PAS (see *lane 6* of Fig. 3) were treated with or without sialidase or mild periodate as described under "Experimental Procedures." Protein A-coated plates were incubated with CD22 mRg, and then incubated with the plasma samples in the absence or presence of α2–6- or α2–3-sLac (1 mM). The plates were incubated with biotin-labeled goat anti-human μ-chain specific Ab, and binding was assayed using peroxidase-conjugated streptavidin. The data are the mean ± S.E. of triplicates from a representative experiment (n = 2).

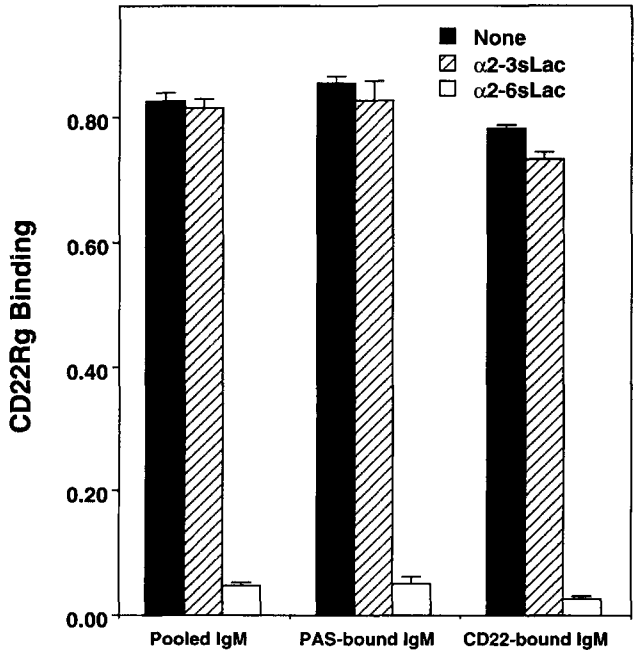

FIG. 5. **Binding of CD22Rg to purified pooled IgM.** PAS-unbound IgM and CD22-bound IgM were prepared from pool IgM as described under "Experimental Procedures." All three IgM preparations were coated in 96-well plates, incubated with CD22 mRg in the absence or presence of α2–6- or α2–3-sLac (1 mM), and assayed as described in the legend to Fig. 1. The data are the mean ± S.E. of triplicates from a representative experiment (n = 2).

binding to TNF-α-activated HEC, with an IC_{50} of 170 ± 44 μg/ml (three experiments), which corresponds to an effective sialic acid concentration of 2.9 ± 0.7 μM (the sialic acid content of purified IgM was measured at 16.8 nmol/mg). Pretreatment of the IgM with mild-periodate/$NaBH_4$ reduction (to selectively truncate sialic acid side chains, leaving the rest of the molecule

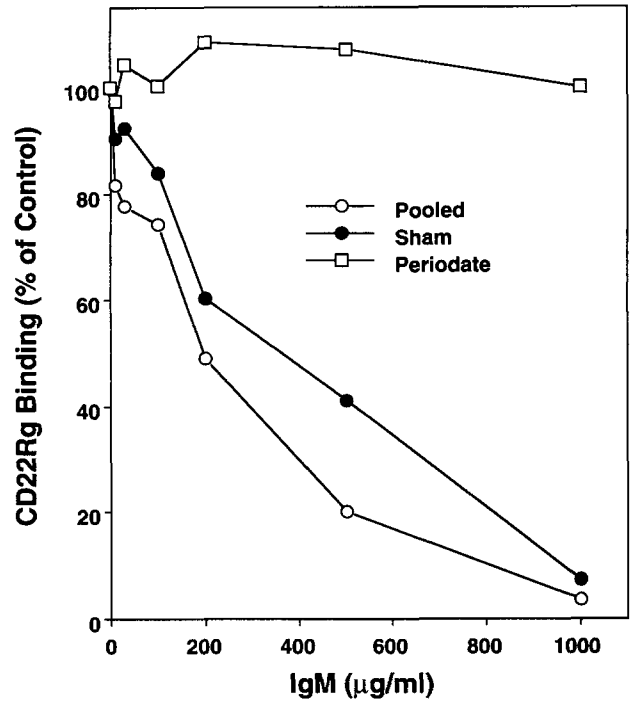

FIG. 6. **Effects of mild periodate oxidation on the ability of IgM to inhibit CD22Rg binding to TNF-α-activated HEC.** TNF-activated HEC were incubated with CD22 mRg in the presence of various concentrations of pooled IgM, sham-treated IgM or mild periodate-treated IgM, and binding assayed as described in the legend to Fig. 1. The data are the mean of duplicates from a representative experiment (n = 2).

intact) completely abolished CD22Rg binding activity (data not shown), as well as its inhibitory effects on CD22Rg binding to TNF-α-activated HEC (Fig. 6). Pretreatment of IgM with proteinase K destroyed its pentameric structure (see "Experimental Procedures") and caused a marked decrease of its inhibitory effects on CD22Rg binding (Fig. 7), shifting the IC_{50} to ~1 mg/ml. Thus, the inhibitory properties of IgM cannot be explained on the basis of sialic acid content alone. In keeping with this, reduction and alkylation of IgM into its component subunits gave a similar reduction of its inhibitory potency (Fig. 8). Commercial pooled haptoglobin also suppressed CD22Rg binding to HEC (72 ± 3% inhibition at 1 mg/ml), whereas IgG showed no inhibition at the same concentration (data not shown). Further studies with haptoglobin were not pursued because of problems with the purity of the samples (including some contamination by IgM).

DISCUSSION

The preceding paper (9) shows that activated vascular endothelium expresses increased levels of CD22-ligands bearing α2–6-linked sialic acids. Such activated endothelial cells are potentially in a position to bind CD22-positive B lymphocytes present in the bloodstream. However, such binding *in vivo* would have to occur in the presence of human blood plasma, which has a high concentration of soluble sialoglycoproteins. Indeed, whole human plasma is shown here to have potent inhibitory properties with regard to CD22 lectin function. Since isolated CD22 is capable of binding to all blood cell types under serum-free conditions (6), this inhibitory property of plasma might also be important to prevent clumping of B cells with other cells in the bloodstream. In this regard, it is noteworthy that the concentrations of many plasma glycoproteins (particularly large molecules such as IgM) are considerably lower in extracellular fluid than in plasma (32). Thus some interactions

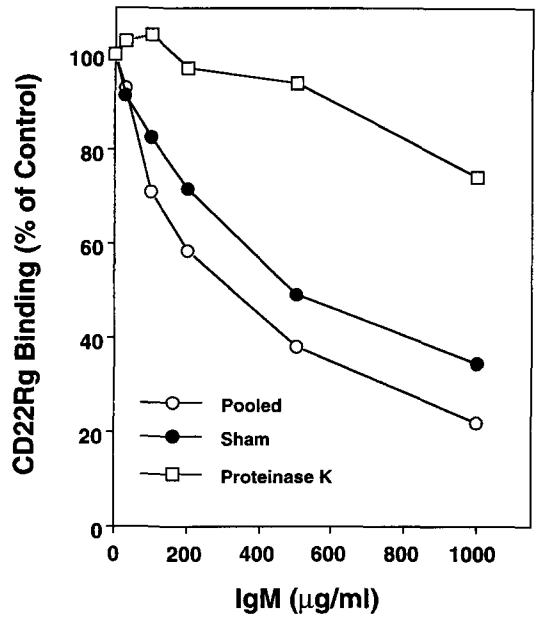

FIG. 7. **Effect of proteinase K digestion on the ability of IgM to inhibit CD22Rg binding to TNF-α-activated HEC.** TNF-α-activated HEC were incubated with CD22 mRg in the presence of various concentrations of pooled IgM, sham-treated IgM, or proteinase K-treated IgM, and binding assayed as described. The data are the mean of duplicates from a representative experiment (n = 2). Diisopropyl fluorophosphate-treated proteinase K alone did not affect the control binding (not shown).

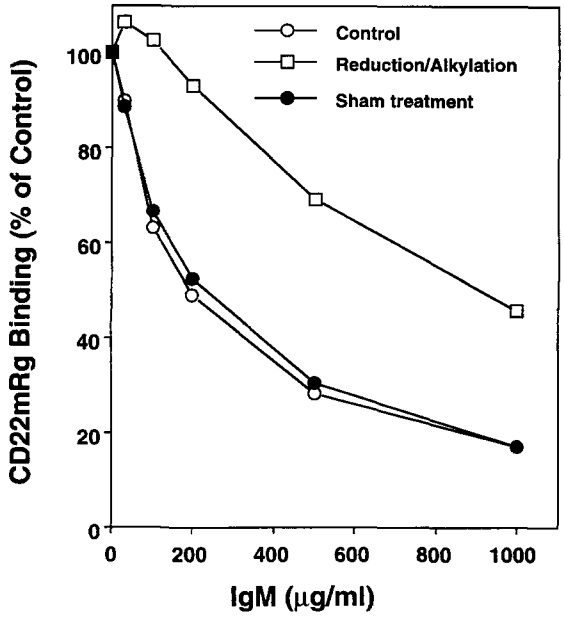

FIG. 8. **Effects of reduction and alkylation on the ability of IgM to inhibit CD22Rg binding to TNF-α-activated HEC.** TNF-α-activated HEC were incubated with CD22 mRg in the presence of various concentrations of pooled IgM, sham-treated IgM, or dithiothreitol/iodoacetamide-treated IgM, and binding assayed as described. The data are the mean of duplicate determinations.

of CD22-positive lymphocytes that are inhibited in the bloodstream might be permitted within lymphoid tissues.

Review of the Carbbank data base (22) indicates that many plasma glycoproteins carry multiple *N*-linked oligosaccharides with α2–6-linked sialic acids, including fibrinogen (normal range 2–4.5 mg/ml), transferrin (2–4 mg/ml), α_2-macroglobulin (1.5–4.0 mg/ml), haptoglobin (1–2.5 mg/ml), α_1-acid glycoprotein (0.5–1.4 mg/ml), IgM (0.6–2.5 mg/ml), hemopexin (0.5–1.2 mg/ml), α_1-antichymotrypsin (0.3–0.6 mg/ml), ceruloplasmin (0.2–0.6 mg/ml), plasminogen (0.1–0.3 mg/ml), antithrombin III (0.17–0.3 mg/ml), and the C1q component of complement (0.1–0.2 mg/ml). Each of these sialoglycoproteins thus has the possibility of having specific interactions with CD22. This study demonstrates that of all of these potential ligands, IgM and haptoglobin can selectively bind to CD22 under conditions where the others do not.

Previously, we demonstrated that certain purified serum glycoproteins including transferrin and fetuin (a fetal bovine serum glycoprotein) can interact with CD22Rg in a Sia-dependent manner (13). However, for both of these proteins, the binding was weak enough that they could be completely removed from CD22Rg-PAS by repeated washing. Somewhat stronger interactions were seen with α_1-acid glycoprotein, with approximately one-third of some batches of this protein surviving repeated washings after binding by CD22Rg-PAS. However, despite its presence in normal plasma at a concentration of 0.5–1.4 mg/ml, α_1-acid glycoprotein was not detected in the present study of total plasma proteins that bound and eluted from CD22Rg-PAS (Fig. 3). This may be because total plasma glycoproteins contain ~1 mM α2–6-Sia residues, which would act as an inhibitor of the binding of molecules with moderate affinity. Indeed, whole plasma blocks CD22Rg-HEC binding with an IC_{50} of 3.7 volume % (corresponding to ~40–50 μM α2–6-Sia residues), which is similar to the IC_{50} for α2–6-sLac in the enzyme-linked immunosorbent assay (30–120 μM) and the K_d of CD22Rg-α2–6-sLac binding (32 μM, see accompanying paper (8)). Thus, the total content of α2–6-Sia residues present on multiple plasma glycoproteins may be sufficient to prevent (or reduce to below the level of detection) the binding of α_1-acid glycoprotein, explaining its absence here.

Given the presence of this high level of "low-affinity" inhibitors in plasma, it is all the more remarkable that IgM and haptoglobin are able to bind selectively to CD22Rg-PAS. The binding of these two "high-affinity" ligands to CD22 clearly depends upon α2–6-linked Sia residues of their carbohydrate moieties, since α2–6-sLac, but not α2–3-sLac, can suppress the interaction. In addition, pretreatment of IgM with sialidase or mild periodate oxidation (which selectively truncates the exocyclic side chain of sialic acids) completely abolishes its CD22 binding activity. IgM also inhibits CD22Rg binding to the ligands on TNF-α-activated HEC in a sialylation-dependent manner, and is at least 18-fold more effective than whole plasma even when considered in relation to its protein-bound sialic acid concentration. Indeed, 50% inhibition of binding was seen with ~0.2 mg/ml pooled IgM, which is well within its physiological concentration range.

Additional structural features of IgM seem to be responsible for its high affinity CD22Rg binding. Extensive destruction of the polypeptide by proteinase K, or mere dissociation of subunits by reduction and alkylation under nondenaturing conditions significantly decreased the ability of IgM to bind to CD22Rg (as judged by its inhibition of CD22Rg-HEC binding). Thus, while α2–6-linked sialic acid residues and their side chains are essential for recognition by CD22, structural features dependent upon the pentameric structure of the $(IgM)_5$·J chain complex are essential for its high affinity binding. Less information is available on the structure of haptoglobin, and given its propensity for variable sialylation and the impurity of different commercial preparations, we did not study it as extensively as IgM. However, haptoglobin also exists in a polymerized form with a high molecular mass in plasma. Thus, for both pentameric IgM and haptoglobin, high-affinity CD22Rg binding may be dependent upon the presentation of multiple sialylated *N*-linked chains in a specific orientation or confor-

mation, with or without participation of additional protein-protein binding. The latter would be analogous to the recently reported differential recognition of various glycoproteins by the mammalian lectins, mannan binding protein and conglutinin (33). In keeping with this possibility, some other multimeric plasma proteins with α2–6-linked sialic acids (such as fibrinogen) were not seen to bind. Regardless, even if IgM and haptoglobin are simply providing a multivalent presentation of α2–6-linked sialic acids, the fact that a subset of cell-surface CD22 exists in a multimerized form (8) gives such a mechanism the potential to be biologically relevant.

While we have not directly measured the K_d values of the binding of either of these glycoproteins to CD22Rg, CD22Rg-HEC binding is blocked by pentameric IgM with an IC_{50} corresponding to ~3 μM α2–6-linked Sia residues, while α2–6-sLac blocks the same interaction with an IC_{50} of ~80 μM. The IC_{50} values measured in solid phase binding assays do not reflect the true solution phase binding constants (8). Since the directly measured K_d of CD22Rg for α2–6-sLac is ~15–30 μM(8), the actual K_d of pentameric IgM-CD22Rg binding may be considerably better than 3 μM.

IgMs are large multimeric glycoproteins with about 10% carbohydrate that carry more sialic acid than IgG, IgA, and IgE (34). Each μ-heavy chain has five *N*-glycosylation sites to which two types of *N*-linked oligosaccharides (high mannose and complex) can be attached, whereas the light chains usually lack glycosylation (35, 36). Structural analyses of oligosaccharides have shown that each pentameric molecule of IgM possesses more than 15 residues of sialic acids, all of which are in the α2–6 linkage on biantennary chains (35–37). Notably, even the *N*-linked oligosaccharides on the J chain of IgM carry such α2–6-sialylated oligosaccharides (38). This may be explained by the finding that B cell activation (which occurs prior to the onset of IgM secretion) (39) is accompanied by up-regulation of the β-galactoside α2,6-sialyltransferase (19, 20), which is known to have a B cell-specific promoter (20, 40).

It is particularly interesting that of all the plasma sialoglycoproteins it is IgM, the major downstream product of B cell *activation* (39), that is capable of binding selectively to a cell-surface receptor of *resting* B cells. Thus, although this interaction was discovered serendipitously while exploring the inhibitory effects of plasma on CD22 interactions with endothelial cells, it might well be that its functional significance is in a different arena. Indeed, it is tempting to speculate that the soluble IgM pentamer might be part of a feedback loop, multivalently cross-linking CD22 molecules to regulate antigen-induced responses and/or B cell aggregation in lymphoid tissues. In this regard, it is noteworthy that the IgM concentration in lymph (and presumably in lymphoid tissues) is estimated to be about 20–45% of the serum concentrations (32), which may be still within the effective range found here. Moreover, local and regional concentrations could be higher or lower in subcompartments of the immune system. Finally, recent studies have indicated an association of CD22 with membrane-bound IgM within the plasma membrane of resting B cells (41, 42), and the consequent tyrosine phosphorylation of CD22 is thought to be involved in antigen-induced cell activation (42, 43). The possibility that this association is also mediated by α2–6-linked sialic acid on membrane IgM must be considered.

In this study, we have focused mainly upon the interactions of IgM with CD22. The interaction with haptoglobin also deserves further exploration. The latter is the major hemoglobin-binding protein of plasma, and is primarily produced by the liver (25, 26). The carbohydrates found on the β-chain constitute about 20% of total molecular mass and the ratio of α2–6- and α2–3-linked sialic acid is about 4:1 (26). Haptoglobin is well-known as a classic "acute phase reactant" (44), whose concentration is substantially elevated in certain inflammatory states (24). Notably, hepatic expression of the sialyltransferase β-galactoside α2,6-sialyltransferase is also elevated under these circumstances (45). Further studies of the potential role of the CD22-haptoglobin interaction in regulating B cell biology during inflammation are required. It also remains to be seen if the lower affinity sialoglycoproteins ligands in the plasma are of biological relevance.

Finally, while CD22 shows exquisite specificity for the primary oligosaccharide sequence that it recognizes, it functions under markedly different conditions than do other vertebrate lectins such as the asialoglycoprotein receptor (46), the mannose 6-phosphate receptors (47), and the hepatic receptor for the sulfated oligosaccharides of pituitary hormones (48). In all these instances, the cognate ligands are relatively rare components among a large excess of other non-competing glycoproteins. In the case of CD22, the primary structural motif recognized is a very common sequence found on the majority of glycoproteins that it encounters. A challenge for the future is to understand how the lectin property of CD22 can mediate specific biological functions (presumably mediated by high affinity ligands) in the midst of a large excess of low affinity ligands. In this regard, it seems important to focus attention upon the ligands with the highest apparent affinity, such as the two reported here.

Acknowledgments—We acknowledge Tom Kipps, Gregg Silverman, Graham Long, and Nissi Varki for helpful comments and for their review of this manuscript.

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