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# Enhanced interaction of L-selectin with the high endothelial venule ligand via selectively oxidized sialic acids

(leukocyte homing/periodate oxidation/endothelium)

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**ABSTRACT** **The selectins are adhesion receptors that play key roles in leukocyte trafficking. Each has an N-terminal C-type lectin domain that binds to specific carbohydrates in a calcium-dependent manner. L-selectin recognizes sulfated, sialylated ligands on lymph node high endothelial venules. This recognition is abolished by strong periodate oxidation under conditions that destroy oligosaccharides. In contrast, mild periodate oxidation, which selectively oxidizes the side chain of sialic acid residues without affecting the underlying oligosaccharide, markedly enhances this interaction. The enhancement is calcium dependent, indicating that lectin recognition is maintained. Reduction of the sialic acid aldehydes generated by mild periodate to alcohol groups abolishes this effect. Covalent cross-linking of the oxidized ligand to L-selectin can be demonstrated, suggesting Schiff base formation between lysine residues of the selectin and the newly formed aldehydes. Such selectively oxidized sialylated ligands could be used to probe the lectin domains of the selectins and to identify lysine residues near the binding site. Also, this approach could be used to design drugs for disrupting leukocyte–endothelial interactions leading to pathological inflammation.**

The selectins are a family of adhesion receptors involved in leukocyte migration into lymphoid tissues and areas of inflammation (1–7). Each selectin has an N-terminal C-type lectin domain that binds specific carbohydrate ligands in a calcium-dependent manner. These domains also contain 10–14 lysine residues (8), which may be involved in ligand binding. Naturally occurring selectin ligands contain sialylated fucosylated lactosamine-type oligosaccharides (9–11). Sulfation of these and/or other oligosaccharides may also be involved in binding (7, 9, 12–15). Two specific ligands have been more precisely identified. The L-selectin binds certain sulfated and sialylated glycoproteins from lymph node high endothelial venules (HEVs) (13, 16), and P-selectin binds a minor 120-kDa neutrophil sialoglycoprotein (17). In both instances, sialic acids are absolutely required for binding.

Periodate oxidation has been used to destroy carbohydrates and to demonstrate biological functions, including lymphocyte–endothelium recognition (18). Such studies used strong periodate oxidation (e.g., 50 mM; pH 5.0), which causes cleavage between *cis*-diols throughout oligosaccharides and destroys them. In contrast, mild periodate oxidation (e.g., 1–2 mM; pH 7.0) selectively oxidizes only the side chain of terminal sialic acids (19–22), leaving the rest of the oligosaccharide intact. Conditions can be adjusted to selectively eliminate only the C9 carbon or both the C8 and C9 carbons (Fig. 1, structures **B** and **C**). The resulting aldehyde can be reduced, leaving a truncated side chain with a primary alcohol group (Fig. 1, structures **D** and **G**). These reactions have been used to explore the importance of the sialic acid side chains in interactions involving viral hemagglutinins, lectins, and antibodies (23–26). Here we have applied them to study the L-selectin–HEV ligand interaction.

## MATERIALS AND METHODS

**Preparation and Purification of the L-Selectin Receptor Globulin.** The L-selectin chimeric molecule contains the entire extracellular domain of the L-selectin (Leu-8) attached to an IgG2 Fc C-terminal domain. cDNAs encoding Leu-8 and IgG2 Fc regions were obtained from human peripheral blood lymphocyte (PBL) total mRNA by PCR amplification. The Leu-8 cDNA was truncated, eliminating the transmembrane domain. A Leu-8/IgG2 Fc chimera was generated in the vector pcDNA1 and stably expressed in 293 cells with G418 selection. Secreted chimera was purified on protein A-Sepharose. This construct is similar to that previously reported (15, 27) and is referred to as L-selectin receptor globulin (LS-Rg).

**Periodate Treatment and LS-Rg Immunohistological Studies.** Freshly cut frozen sections of rat cervical lymph nodes (27) were blocked with 10% goat serum/1% bovine serum albumin in Hanks' balanced salt solution containing 1 mM $Ca^{2+}$ (HBSS) (4°C; 20 min). Prior to overlaying with human PBLs (28) or LS-Rg, sections were exposed to sodium metaperiodate for 30 min at the concentrations, temperatures, and pH values noted. Slides were incubated sequentially as indicated with one wash in HBSS between incubations. After the final wash, LS-Rg was detected by sequential incubations with biotinylated goat anti-human IgG and avidin-peroxidase [both in blocking buffer; 30 min; room temperature (RT)], development with 3-amino 9-ethylcarbazole-peroxide, and hematoxylin counterstaining.

**Labeling of Ligand from Lymph Node Sections.** A major ligand for L-selectin is shed into the medium of lymph nodes in organ culture (16). Preparations enriched in this ligand were made by incubating minced murine lymph nodes in sulfate-deficient medium supplemented with $Na^{35}SO_4$ for 18 hr. Culture supernatants were clarified by ultracentrifugation and passed over a wheat germ agglutinin (WGA) column (16). This preparation was used without further purification; 40–50% of this material was capable of binding to LS-Rg.

**Periodate Treatment and Reduction of $^{35}$S-Labeled Ligand.** WGA-enriched $^{35}SO_4$-labeled material was treated with 2 mM sodium metaperiodate in phosphate-buffered saline (pH 7.0) (4°C; 30 min) in the dark, followed by dialysis against 20 mM Mops, pH 7.4/100 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/0.02% azide/0.01% Triton X-100 in a microdialyzer (five

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Abbreviations: HEV, high endothelial venule; PBL, peripheral blood lymphocyte; LS-Rg, L-selectin receptor globulin; hIgG, human IgG; RT, room temperature; WGA, wheat germ agglutinin.

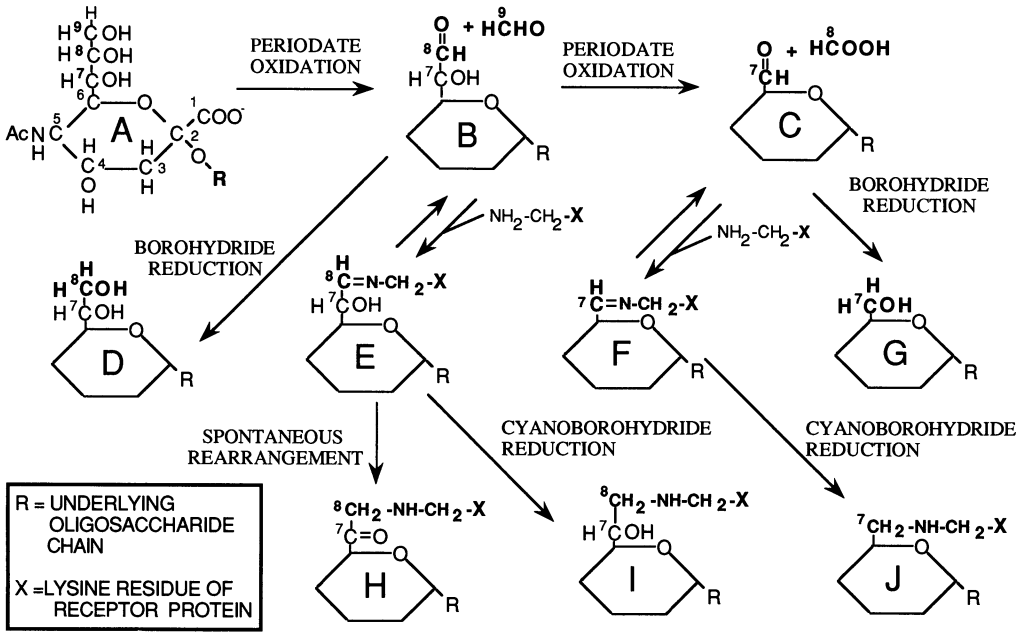


FIG. 1. Chemical reactions involving the side chain of sialic acids. Structure **A** shows *N*-acetylneuraminic acid, the most common sialic acid molecule, in $\alpha$ linkage to an underlying oligosaccharide, designated R. Various reactions referred to in the text are outlined. Only the relevant portions of each molecule are shown in complete form for structures **B–J**.

exchanges of 100 ml). Aliquots of treated or sham-treated material were incubated with LS-Rg or control human IgG (hIgG) in 3.9 mM $Ca^{2+}$ or 7.9 mM EDTA at RT. After 30 min, 200 mM sodium cyanoborohydride was added and incubated for 1 hr. The LS-Rg or hIgG was precipitated using an excess (20 $\mu$l) of protein A-Sepharose, and the bound and unbound materials were examined by SDS/7.5% PAGE (under reducing conditions) and fluorography.

## RESULTS

Using mild periodate and borohydride reduction, it has been shown that truncation of the sialic acid side chains does not destroy recognition by E-selectin (14). We examined this question with regard to L-selectin, using the Stamper–Woodruff assay, in which human PBLs are layered over cryosections of lymph nodes, and their binding to HEVs is evaluated (29). In this assay, binding is L-selectin dependent and is cross-reactive between tissues and cells from different species (30). Since control borohydride treatment of sections caused some nonspecific inhibition of PBL binding, we studied the effect of periodate alone. In keeping with previous reports (18), we observed $Ca^{2+}$-dependent binding, which was abolished by strong periodate oxidation. In contrast, mild periodate treatment did not abolish binding but, in fact, enhanced it (Fig. 2).

To avoid potential complexities from other adhesion molecules that can come into play in PBL–HEV adhesion, we turned to immunohistochemical staining of lymph node cryosections using the LS-Rg chimera. Tissue sections were exposed to various treatments before or after binding of LS-Rg (Table 1 and Fig. 3). In agreement with others (27), calcium-dependent staining of HEVs was blocked by EDTA. Mild periodate treatment markedly enhanced calcium-dependent staining (Fig. 3, compare *Upper Left* and *Lower Left*). When EDTA was added after LS-Rg incubation, binding was completely dissociated in nontreated sections but only partially dissociated in the mild periodate-treated sections (Fig. 3 *Right*). When mild periodate treatment was followed by borohydride reduction, staining returned to control levels, indicating that the enhanced binding is not due to side-chain truncation but requires the aldehyde groups. Also, as seen with PBL binding, strong periodate oxidation abolished binding of LS-Rg to HEVs. Since strong periodate oxidation should produce at least as many aldehyde groups as generated by mild periodate, the enhanced staining seen with mild periodate cannot be due simply to generation of aldehyde groups on HEVs. These data indicate that aldehyde groups generated on sialic acid side chains of the HEV ligand are specifically required for the enhanced interaction.

We hypothesized that the sialic acid aldehyde groups on the HEV ligand might form Schiff bases with lysine amino

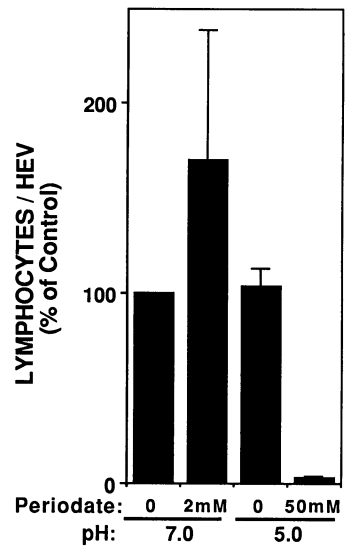


FIG. 2. Effects of mild and strong periodate oxidation on lymphocyte binding to HEVs. Fixed cryostat sections of rat lymph nodes were overlaid with freshly isolated human PBLs and their binding to HEVs was monitored (26–28). Prior to this, some sections were exposed to periodate under the conditions indicated. Lymphocytes attached to 20–40 HEVs per section were counted and averaged. Data are presented relative to results with pH 7.0 buffer at 4°C (expressed as 100%) and are means ± SD from five experiments at pH 7.0 and from three experiments at pH 5.0.

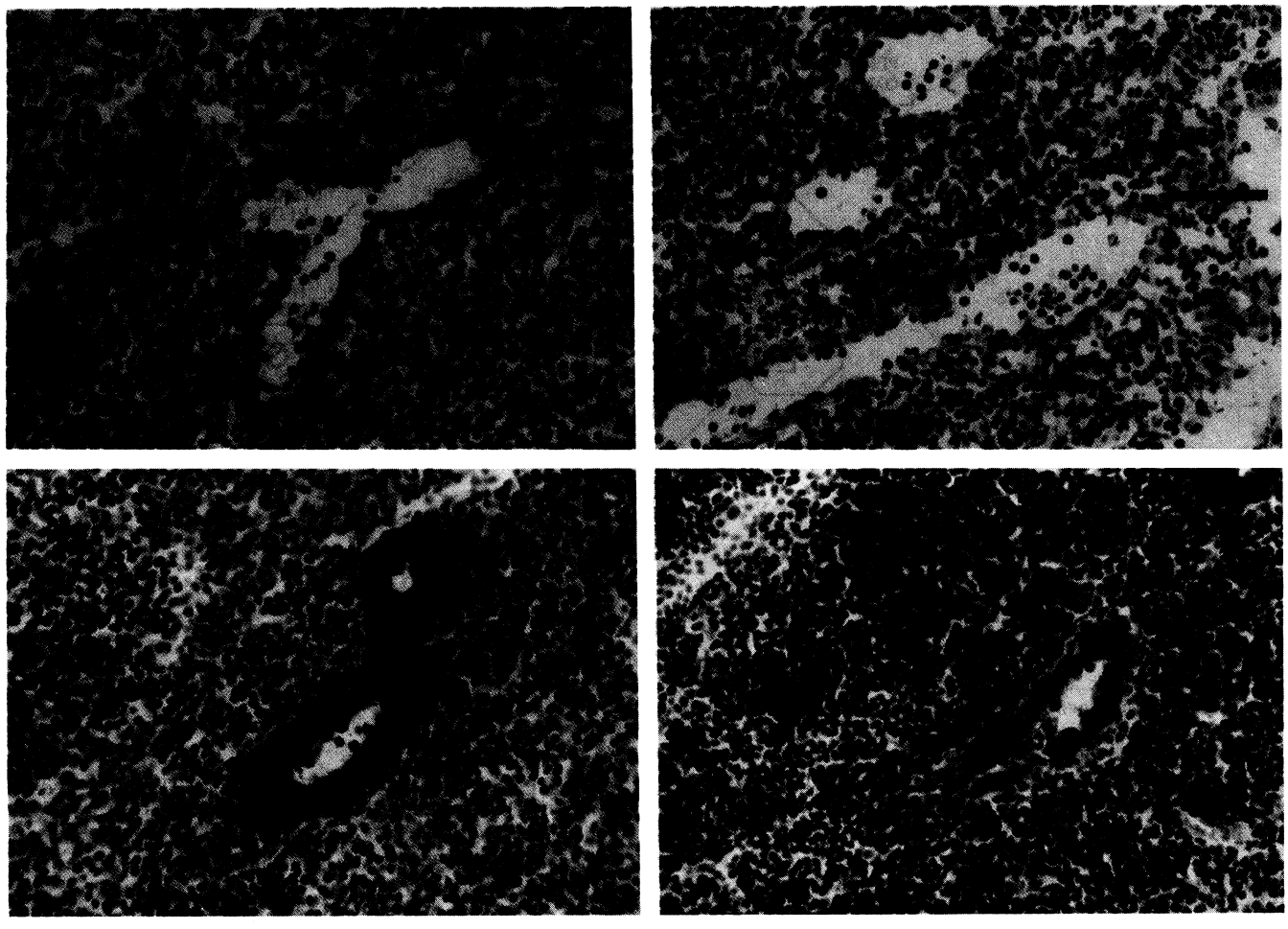

FIG. 3. Effects of mild periodate on LS-Rg staining of HEVs. Fixed cryostat sections of rat lymph nodes were overlaid with the LS-Rg. Sections were exposed to various treatments before or after LS-Rg binding (27); details are provided in Table 1. (*Upper Left*) Control (no periodate, no EDTA). (*Lower Left*) Mild periodate (2 mM) prior to LS-Rg. (*Right*) Same as *Left* except that LS-Rg binding was followed by EDTA (20 min; RT) prior to staining. Amount of LS-Rg used was set in the control to clearly emphasize the enhancement by mild periodate treatment. (Bar = 50 $\mu$m.)

groups in the lectin domain of the selectin and thus enhance binding (Fig. 1, structures **E** and **F**). Although covalent, Schiff bases are slowly reversible unless they undergo spontaneous rearrangement to form more stable Amadori compounds (31). For oxidized sialic acids, rearrangement is possible only with the 8- and not with the 7-carbon aldehyde form (Fig. 1, structure **H**). Under the conditions used in Fig. 3 (excess periodate), the 7-carbon form should predominate. In fact, prolonged incubations in EDTA after LS-Rg binding almost completely reversed the enhanced staining with mild periodate (data not shown). These findings support the hypothesis that reversible Schiff bases are involved in the enhanced interaction.

Schiff bases can be irreversibly reduced to stable secondary amines with cyanoborohydride (32, 33) (Fig. 1, structures **I** and **J**). To demonstrate covalent linkage between the L-selectin and its oxidized ligand, we used the WGA-enriched $^{35}SO_4$-labeled ligand from lymph nodes (13). The LS-Rg-$^{35}$S-labeled ligand complex can be precipitated by protein A-Sepharose (Fig. 4*A*; note that only half of the WGA-purified material can be precipitated by the LS-Rg). Treatment of this ligand with mild periodate did not alter the amount precipitated by LS-Rg (data not shown). However, when the complexes were exposed to cyanoborohydride prior to protein A binding, different results were obtained (Fig. 4*B*). Upon reduction of these periodate oxidized ligands plus LS-Rg, a significant portion of the label remained in the pellet and migrated at higher molecular weights on SDS/PAGE (Fig. 4*B*). The bands probably represent various ratios of covalently bound ligand to LS-Rg, since both are polydisperse glycoproteins. Formation of these covalent adducts was calcium dependent, and adducts were not seen with control hIgG in place of LS-Rg. For uncertain reasons, cyanoborohydride treatment itself disrupted the native LS-Rg–ligand complex. Thus, in controls, all radioactivity was recovered in the supernate, and LS-Rg remained in the pellet (by Coomassie staining; data not shown).

## DISCUSSION

The selectins play critical roles in initiating interactions between leukocytes and endothelial cells during normal acute and chronic immune responses (1–6, 30). They also participate in disorders in which leukocytes cause tissue damage, such as postperfusion injury, acute respiratory distress syndrome, and some chronic inflammatory disorders (1–6, 30, 34–36). They may also be important in the process of tumor metastasis (37, 38). Thus, there is considerable interest in understanding the mechanisms of selectin function and in developing compounds that can selectively block selectin interactions *in vivo*.

We have presented data indicating that truncation of the sialic acid side chain(s) on an L-selectin ligand does not prevent its calcium-dependent binding by the selectin. However, once bound, the aldehyde group and a nearby lysine group on the selectin presumably form a Schiff base, which

Table 1. Effects of various sequential treatments on binding of LS-Rg to HEVs

| Sequential incubation number | | | | | | |
|---|---|---|---|---|---|---|
| First | | Second | Third | | Fourth | |
| pH | Periodate, mM | Borohydride (22°C) | L-selectin chimera | EDTA | EDTA (22°C) | Relative staining |
| 7.0 | — | − | − | − | − | 0 |
| 7.0 | — | − | + | − | − | 1–2+ |
| 7.0 | 2 | − | + | − | − | >4+ |
| 7.0 | 2 | + | + | − | − | 1+ |
| 7.0 | — | + | + | − | − | 1+ |
| 7.0 | — | − | + | + | − | 0 |
| 7.0 | 2 | − | + | + | − | ± |
| 7.0 | — | − | + | − | + | 0 |
| 7.0 | 2 | − | + | − | + | 3–4+ |
| 5.0 | — | − | − | − | − | 0 |
| 5.0 | — | − | + | − | − | 1–2+ |
| 5.0 | 50 | − | + | − | − | ± |

Freshly cut frozen sections of rat cervical lymph nodes were fixed and prepared as described (27). Slides were incubated sequentially as indicated. First incubations: 4°C for 30 min HBSS (pH 7.0) or 50 mM sodium acetate, pH 5.0/100 mM NaCl, with or without sodium metaperiodate (prepared fresh) at the final concentrations indicated. Second incubations: RT for 10 min in phosphate-buffered saline with or without freshly prepared sodium borohydride (final concentration, 20 mM). Third incubations: 4°C for 30 min in HBSS with or without LS-Rg (10 μg/ml) and with or without EDTA (10 mM; 10-fold excess over $CA^{2+}$ in HBSS). Fourth incubations: RT for 20 min in HBSS with or without 10 mM EDTA. Binding of LS-Rg was detected as described. Relative staining is reported on a qualitative scale of 1 to 4+ (see Fig. 3 for examples). In some cases (±), only trace variable staining was seen. Results represent summary of >10 separate experiments.

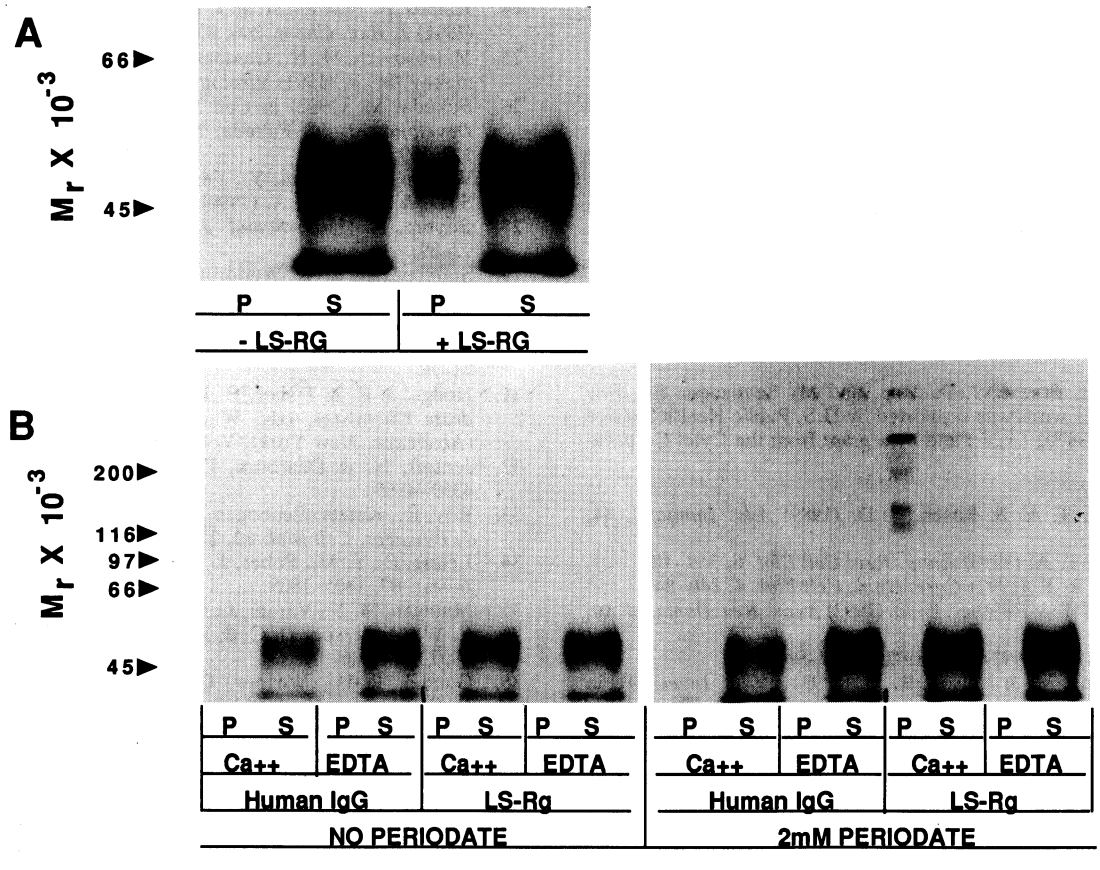


FIG. 4. Evidence for calcium-dependent covalent binding of LS-Rg to $^{35}SO_4$-labeled ligand from lymph nodes. (*A*) WGA-enriched $^{35}SO_4$-labeled material from lymph node culture supernatant was incubated with or without LS-Rg (4°C; 14–15 hr) and the chimera was quantitatively precipitated by excess protein A-Sepharose. The supernatant fluid (S) and the pellet (P) were lyophilized, boiled in sample buffer, and subjected to SDS/7.5% PAGE and fluorography. Only a portion of the WGA eluate material can bind to LS-Rg (which is used in excess). (*B*) WGA-enriched $^{35}SO_4$-labeled sialylated material from lymph nodes was sham-treated or treated with 2 mM sodium metaperiodate, incubated with LS-Rg or hIgG in $Ca^{2+}$ or EDTA, and reduced with sodium cyanoborohydride; globulins were precipitated with protein A-Sepharose and analyzed as in *A*. Coomassie staining prior to fluorography showed that LS-Rg or hIgG had been precipitated by protein A (data not shown). The ligand was nonspecifically displaced from LS-Rg by cyanoborohydride except when mild periodate pretreatment was done.

can be reduced to a stable covalent bond with cyanoborohydride. Such adducts could help to identify critical lysine residues at or close to the lectin binding pocket and to determine the number of binding sites per selectin.

Small soluble oligosaccharides (e.g., the sialyl-Lewis$^x$ tetrasaccharide) have been shown to block binding, but high concentrations ($\mu$M to mM range) are required, and rapid renal clearance may make it difficult and/or expensive to sustain effective therapeutic levels. Binding may be blocked better with multimeric glycopeptides with better affinities and longer circulating half-lives. However, such compounds may cause unwanted intercellular adhesion, and enhance the possibility of immune reactions to the sugar chains. We suggest that monomeric ligands with oxidized side chains could be effective inhibitors transiently inactivating selectin molecules until new ones are synthesized. These would require only a short exposure with adequate concentrations and would have minimal immunogenicity. Finer regulation might be obtained with various ratios of the 7-carbon and the 8-carbon aldehyde form (which would vary the likelihood of Amadori rearrangement and stabilization under physiological conditions). Of course, all such approaches require that nonspecific interactions between the aldehydes and lysine groups on plasma proteins be minimal. In this regard, our *in vitro* studies do not show measurable binding to hIgG, or to bovine serum albumin, a lysine-rich protein, under conditions in which substantial binding to L-selectin occurs (data not shown).

Since L-selectin ligand(s) can be obtained only in pmol quantities (27), we have not been able to isolate and directly characterize the covalent linkage. However, we can think of no explanation for our findings other than Schiff base formation. Future work with larger quantities of natural or synthetic ligands will be needed to conclusively prove that Schiff base formation is indeed the cause of enhanced binding. While prior studies indicated that the sialic acid side chain is not important for E-selectin binding (14), the aldehydes were reduced before analyzing binding, leaving no possibility for generating Schiff bases. Exploration of the interaction of aldehyde-bearing ligands with the P- and E-selectins to determine whether the phenomenon seen with L-selectin is applicable to the other two molecules remains to be done.

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