Endothelial Targeting and Enhanced Antiinflammatory Effects of Complement Inhibitors Possessing Sialyl Lewis\(^x\) Moieties


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Endothelial Targeting and Enhanced Antiinflammatory Effects of Complement Inhibitors Possessing Sialyl Lewis\textsuperscript{x} Moieties\textsuperscript{1}

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The complement inhibitor soluble complement receptor type 1 (sCR1) and a truncated form of sCR1, sCR1[desLHR-A], have been generated with expression of the selectin-reactive oligosaccharide moiety, sialyl Lewis\textsuperscript{x} (sLe\textsuperscript{x}), as N-linked oligosaccharide adducts. These modified proteins, sCR1sLe\textsuperscript{x} and sCR1[desLHR-A]sLe\textsuperscript{x}, were assessed in the L-selectin- and P-selectin-dependent rat model of lung injury following systemic activation of complement by cobra venom factor and in the L-selectin-, P-selectin-, and E-selectin-dependent model of lung injury following intrapulmonary deposition of IgG immune complexes. In the cobra venom factor model, sCR1sLe\textsuperscript{x} and sCR1[desLHR-A]sLe\textsuperscript{x} caused substantially greater reductions in neutrophil accumulation and in albinum extravasation in lung when compared with the non-sLe\textsuperscript{x}-decorated forms. In this model, increased lung vascular binding of sCR1sLe\textsuperscript{x} and sCR1[desLHR-A]sLe\textsuperscript{x} occurred in a P-selectin-dependent manner, in contrast to the absence of any increased binding of sCR1 or sCR1[desLHR-A]. In the IgG immune complex model, sCR1[desLHR-A]sLe\textsuperscript{x} possessed greater protective effects relative to sCR1[desLHR-A], based on albumin extravasation and neutrophil accumulation. Enhanced protective effects correlated with greater lung vascular binding of sCR1[desLHR-A]sLe\textsuperscript{x} as compared with the non-sLe\textsuperscript{x}-decorated form. In TNF-\alpha-activated HUVEC, substantial in vitro binding occurred with sCR1[desLHR-A]sLe\textsuperscript{x} (but not with sCR1[desLHR-A]). This endothelial cell binding was blocked by anti-E-selectin but not by anti-P-selectin. These data suggest that sLe\textsuperscript{x}-decorated complement inhibitors have enhanced antiinflammatory effects and appear to have enhanced ability to localize to the activated vascular endothelium. The Journal of Immunology, 1999, 162: 4952–4959.

Many acute inflammatory reactions are complement dependent. The complement system serves as a source of peptides (anaphylatoxins, C3a and C5a) that have powerful vasopermeability and/or leukocyte chemotactic activities. Inflammatory reactions that are complement dependent can be suppressed by the inhibition of complement activation products or by blocking activation of the complement system. One effective strategy for suppressing these reactions is the blockade of complement activation products (such as C5a) with specific Ab (1–4). These Ab-based approaches, however, have the intrinsic problem of the development of immune responses to idiotypic or nonidotypic domains of the blocking Abs. Blocking activation of the complement system can be achieved by consumptive complement depletion, such as which occurs with repeated i.p. injection of purified cobra venom factor (CVF) (5, 6), by Ab-induced inhibition of individual complement components such as C5 (3), or by infusion of soluble versions of soluble complement receptor type 1 (sCR1) (7).

sCR1 (soluble CD35) is a single chain glycoprotein consisting of 30 homologous protein domains known as short consensus repeats (SCR), followed by transmembrane and cytoplasmic domains (8, 9). Groups of seven SCRs form long homologous repeats (LHRs), which have been designated LHR-A, -B, -C, and -D (10). Three adhesion molecules expressed during endothelial activation of the alternative pathway of complement activation. In the first steps of the inflammatory response, recruitment of leukocytes into tissues requires that the vascular endothelium be “activated” to express adhesion molecules, which serve to tether blood leukocytes to the endothelium before their diapedesis (13–18). Two adhesion molecules expressed during endothelial activation are P- and E-selectin, both containing binding sites for sLe\textsuperscript{x} and related motifs present on neutrophils and other leukocytes (13, 17, 18). Developing molecules to interrupt the binding of leukocytes to endothelial selectins has been a goal of many drug development programs. One particular strategy has been the use of soluble oligosaccharides containing the sLe\textsuperscript{x}
moeity (19). In the current report, we assessed sLe\(^x\)-decorated versions of sCR1 (and the truncated forms). Such altered molecules might possess two novel features. First, they could physically block the initial, selectin-dependent in vivo leukocyte binding (associated with the “rolling” phenomenon) and consequently act as anti-inflammatory agents. Second, the presence of the sLe\(^x\) motif within natural oligosaccharides of sCR1 could also serve to localize this molecule to areas of inflammation by binding to endothelial selectins that are reactive with sLe\(^x\).

One method to obtain proteins with the sLe\(^x\) motif attached to naturally expressed oligosaccharides would be in vitro chemical or enzymatic modification of proteins after their synthesis. This method would likely result in limited amounts of the final desired product. The alternative, chosen for the current studies, would be production of the recombinant protein in a cell line containing the specific fucosyl transferase activity that would allow addition of α1-3 linked fucose during the course of normal oligosaccharide synthesis (20, 21). This would allow production of the sLe\(^x\) motif within natural N-linked oligosaccharides. This strategy was applied for production of modified proteins, sCR1sLe\(^x\) and sCR1[desLHR-A]sLe\(^x\). These proteins have been shown to possess sLe\(^x\) as a portion of their natural N-linked oligosaccharides (48) (M. D. Picard et al., manuscript in preparation). This report describes the in vivo efficacy of sCR1sLe\(^x\) and sCR1[desLHR-A]sLe\(^x\) in lung inflammatory models of neutrophil-mediated lung injury: systemic activation of complement, which induces injury that is P- and L-selectin dependent (22–25), and intrapulmonary deposition of IgG immune complexes, which induces injury that is P-selectin, L-selectin, and E-selectin dependent (23–26). Previously, in both models of lung injury, sCR1 and the soluble sLe\(^x\) tetrasaccharide have been shown to be protective (7, 27, 28). The current studies indicate that sLe\(^x\)-decorated versions of sCR1 are more protective in vivo and have the ability to localize to the activated vascular endothelium.

Materials and Methods
Recombinant complement inhibitory proteins
sCR1 was produced in the Chinese hamster ovary cell line DUKX B11 and purified as previously described (11). sCR1[desLHR-A] was produced in the DUKX B11 cell line that had been transfected with the plasmid pT- CR1c6A. The resulting secreted glycoprotein was purified and characterized. sCR1[desLHR-A] was an effective inhibitor of the alternative complement pathway comparable to sCR1 (see below). As expected, sCR1[desLHR-A] was a less effective in vitro inhibitor of the classical complement pathway when compared with sCR1 (12). sCR1[desLHR-A] and sCR1[desLHR-A]sLe\(^x\) were labeled with \(^{125}\)I [New England Nuclear, Boston, MA] using lactoperoxidase techniques. sCR1 and sCR1[desLHR-A] produced by DUKX B11 possessed no sLe\(^x\) on their N-linked oligosaccharides (48) (M. D. Picard et al., manuscript in preparation). For sCR1sLe\(^x\) production, the expression plasmid coding for sCR1 was used (11). The plasmid pTCSLDHFR\(^B\) coding for a mutant mouse dihydrofolate reductase with an abnormally low affinity for methotrexate (29), was derived from pSV2-DHFR and cloned into pTCSLin2 by direct substitution of the neomycin resistance gene. The Chinese hamster ovary cell line, LEC11 (20), which expresses the dihydrofolate reductase activity, was transfected together with 125I-sCR1[desLHR-A] or 125I-sCR1[desLHR-A]sLe\(^x\). This is when up-regulation of P- and L-selectin is maximal (25). Lung injury was quantitated by permeability and MPO measurements, as indicated above.

Inhibition of complement activation in vitro
Having established the presence of the sLe\(^x\) tetrasaccharide in the LEC11 glycoproteins, as described above, it was important to examine the effects of such glycosylation on complement inhibitory function. As described elsewhere, the concentrations required to inhibit human complement-mediated lysis of erythrocytes were similar for glycoproteins expressed by LEC11 cells (sLe\(^x\) versions) compared with those expressed by DUKX-B11 cells (non-sLe\(^x\) version). Both versions of sCR1 and sCR1[desLHR-A] were similar in their capacity to inhibit alternative pathway of complement activation, but the sCR1 form was, as expected, more effective than sCR1[desLHR-A] in the inhibition of the classical complement pathway. Analogous results were obtained for the two versions of sCR1[desLHR-A]. In the classical pathway assay, sCR1[desLHR-A]sLe\(^x\) (IC50 = 140 ± 32 nM, n = 3) was somewhat less effective than sCR1[desLHR-A] (IC50 = 58 ± 38 nM, n = 4) but much less effective than either version of sCR1. In the alternative pathway assay, sCR1[desLHR-A]sLe\(^x\) (IC50 = 46 ± 9.1 nM, n = 4) was again somewhat less effective than sCR1[desLHR-A] (IC50 = 37 ± 6.2 nM, n = 4) but comparable to either version of sCR1.

In vitro binding assays using endothelial cells
For in vitro binding of sCR1[desLHR-A] or sCR1[desLHR-A]sLe\(^x\) to endothelial cells, freshly isolated HUVEC (passage 1–3) in 12-well tissue culture plates were incubated for 4 h at 37°C in the presence or absence of 50 ng/ml TNF-α. The cells were washed twice with HBSS containing 1% BSA and then incubated with sCR1[desLHR-A] or sCR1[desLHR-A]sLe\(^x\) (10 nM) together with trace amounts of 125I-labeled proteins in the presence or absence of Abs (5 μg/ml) directed against human E-selectin (CL-3), P-selectin (PB1.3), or an isotype-matched irrelevant Ab (MOPC-21). Following a 10-min incubation, the
cells were washed to remove any unbound radiolabel and then lysed with 2% Triton X-100 for isotope counting. Nonspecific binding to the cells was measured in the presence of 50- to 100-fold excess of unlabelled sCR1[desLHR-A] or sCR1[desLHR-AjLe].

**Tissue myeloperoxidase content**

Whole lungs were homogenized with a Polytron homogenizer (Tekmar, Cincinnati, OH) (4 to 10 s at setting of 4) in a volume of 6 ml, using a homogenizer buffer (50 mM phosphate, pH 6.0). Samples were then subjected to centrifugation (3000 × g, 30 min) at 4°C. Myeloperoxidase (MPO) activity in supernatant fluid was assayed by measuring the change (per min) in absorbance at 460 nm resulting from the oxidation of o-dianisidine in the presence of H2O2 (7).

**Immunohistochemistry**

For immunostaining of frozen sections, rat lungs were frozen in optimal cutting temperature (O.C.T.) compound (Miles, Elkhart, IN) and stained with rabbit polyclonal IgG Ab to human sCR1, which was diluted 1:1000 in PBS (pH 7.4) containing 0.1% BSA for 1 h in a humidified chamber. Slides were then washed twice in PBS and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG-specific Ab (Rockland, Gilbertsville, IL) diluted 1:10,000 in PBS. Slides were washed twice in PBS, dried, and incubated with horseradish peroxidase-specific substrate True Blue (Kirkegaard & Perry, Gaithersburg, MD) for 5 min, washed, then dipped in 100% ethanol, dried, and mounted under coverslips.

**Statistical analysis**

The data were subjected to ANOVA. Paired or unpaired Student’s t test multigroup comparisons were also determined using the Schaffer t test, as well as the Fisher protected least significant difference test. All statistical comparisons were made between treatment groups and positive controls after mean background negative control values had been subtracted from each data point. All values were expressed as mean ± SEM. Statistical significance was defined as p < 0.05.

**Results**

**CVF-induced lung injury: comparison of the protective effects of sCR1 and sCR1sLe**

The relative efficacy of sCR1 and sCR1sLe was determined in the CVF model of lung injury employing i.v. doses of 0.30, 1.5, and 4.5 mg per animal. Inhibitors (200 μl) were infused i.v. immediately before i.v. infusion of 4 U of CVF together with 5 μCi 125I-BSA (100 μl), which was used to measure lung vascular permeability. Animals were sacrificed 30 min later. Results are shown in Table I. After infusion of CVF, there were 5- to 10-fold increases in albumin leak and in MPO content when compared with negative controls (no CVF) (Table I, footnotes). At doses of 0.30, 1.5, and 4.5 mg, sCR1 reduced permeability indices by 20%, 34%, and 42%, respectively. The latter two values showed statistically significantly greater protection (p < 0.05) when compared with the values of the otherwise untreated positive controls (CVF alone). In the companion group treated with sCR1sLe, the doses of 0.30, 1.5, and 4.5 mg caused reductions of 57%, 69%, and 80%, respectively, all of which were significantly greater than values obtained with sCR1 (Table I). Thus, at similar doses, sCR1sLe was substantially more protective than sCR1.

Lung neutrophil accumulation as defined by lung MPO content was also compared for each of the three doses of the two sCR1 compounds (Table I). At doses of 0.30, 1.5, or 4.5 mg, sCR1 caused reductions in MPO content in lung of 18%, 33%, and 40%, respectively, all being statistically significant (p < 0.05) when compared with the otherwise untreated positive controls. In animals treated with sCR1sLe at doses of 0.30, 1.5, or 4.5 mg, MPO levels fell by 33%, 56%, and 75%, respectively, each being statistically significantly more protective when compared with the effects of sCR1 (Table I). Thus, sCR1sLe is a more effective inhibitor than is sCR1.

The observed differences in the efficacy of sCR1 and sCR1sLe could not be explained by differences in the pharmacokinetic or complement-inhibiting properties of the compounds. The pharmacokinetics of sCR1 and sCR1sLe were determined in normal adult male rats (n = 5 for each group) given a bolus i.v. infusion (10 mg/kg) of either sCR1 or sCR1sLe. Plasma samples (obtained at 0, 3, 10, 30, and 60 min during the first hour) were quantitated for sCR1 and sCR1sLe by enzyme immunoassay techniques. During the first hour, the blood levels of the two compounds were not statistically different (data not shown). It would appear in the CVF model of lung injury (where sacrifice occurs at 30 min) that the greater protective effects of sCR1sLe cannot be explained by differences in pharmacokinetics. With respect to complement-inhibiting properties of sCR1 and sCR1sLe, similar concentrations of sCR1 and sCR1sLe showed similar inhibition in human serum complement-mediated lysis of Ab-sensitized sheep erythrocytes (see above).

**Immunostaining of sCR1 and sCR1sLe in rat lung after CVF infusion**

Using immunostaining to detect binding of sCR1 and sCR1sLe to the lung vasculature, 0.30 mg of each inhibitor was injected i.v. 10 min after i.v. infusion of 4 U of CVF. Animals were then sacrificed 10 min after the infusion of either sCR1 or sCR1sLe, and the lungs were prepared for immunostaining. At this point (20 min after infusion of CVF), lung vascular P-selectin is maximally up-regulated (31). A polyclonal affinity-purified Ab to sCR1 (which also detects sCR1sLe) was employed. The results of these studies are shown in Fig. 1. Rats treated with sCR1 and CVF revealed no detectable binding of sCR1 to the lung vasculature (Fig. 1, A and C), whereas treatment with sCR1sLe and CVF resulted in obvious evidence of binding of sCR1sLe to lung interstitial capillaries and venules (Fig. 1, B and D). These data provide direct evidence for
the binding of sCR1sLe\(^x\), but not sCR1, to the lung vasculature of animals following i.v. infusion of CVF.

Protective effects of sCR1\{desLHR-A\} and sCR1\{desLHR-A\}sLe\(^x\) after CVF infusion

We evaluated the protective effects of sCR1\{desLHR-A\} and sCR1\{desLHR-A\}sLe\(^x\) in the CVF model of lung injury. The results are shown in Table II. At sCR1\{desLHR-A\} doses of 0.30, 1.5, and 4.5 mg, the permeability indices fell by 6%, 41%, and 53%, respectively, the latter two values being statistically significantly different from those of the reference positive control groups not otherwise treated. In animals treated with sCR1\{desLHR-A\}sLe\(^x\), at the same doses, the permeability indices were reduced 20%, 53%, and 69%, respectively. Only the highest dose gave statistically greater protection \((p < 0.03)\) when compared with the undecorated form of the inhibitor.

When MPO measurements were determined, the sCR1\{desLHR-A\} doses of 0.30, 1.5, and 4.5 mg caused reductions of 8%, 34%, and 46%, respectively (Table II). Statistically, when compared with the otherwise untreated positive controls, the two higher doses of sCR1\{desLHR-A\} achieved statistical significance. When animals were treated with sCR1\{desLHR-A\} sLe\(^x\) at doses of 0.30, 1.5, and 4.5 mg, the reductions in MPO content were 22%, 44%, and 66%, respectively. Again, only the highest dose of sCR1\{desLHR-A\} sLe\(^x\) achieved statistical significance \((p = 0.01)\) when compared with sCR1\{desLHR-A\}. Thus, although the sLe\(^x\)-decorated compound was a more effective inhibitor, these effects were only seen at the highest dose, unlike the full-length sCR1s described in Table I.

Binding of sCR1\{desLHR-A\} and sCR1\{desLHR-A\}sLe\(^x\) to lung vasculature after CVF infusion

With availability of \(^{125}\)I-versions of the decorated and undecorated sCR1\{desLHR-A\} compounds, we evaluated the lung vascular binding of \(^{125}\)I-sCR1\{desLHR-A\} and \(^{125}\)I-sCR1\{desLHR-A\}sLe\(^x\) after i.v. infusion of PBS or 4 U of CVF in the presence or absence of 200 \(\mu\)g i.v. infused PB1.3 IgG1 (anti-P-selectin) or MOPC-21 IgG1 (irrelevant control Ab). Twenty minutes later, lung binding was determined in lungs after the pulmonary artery had been infused with sterile saline (10 ml) to clear residual blood. The composite results of these studies are shown in Table III. The binding of sCR1\{desLHR-A\} and sCR1\{desLHR-A\}sLe\(^x\) to the lung vasculature in the presence of circulating MOPC-21 after infusion of either saline was comparable \((5.78 \pm 0.53\) vs \(6.34 \pm 0.715\) \(\mu\)g, respectively; \(p, NS)\). The binding to lung of sCR1\{desLHR-A\}sLe\(^x\) in the presence of MOPC-21 in saline-infused animals was similar to sCR1\{desLHR-A\} to the binding after saline infusion \((6.34 \pm 0.31)\) and in CVF-infused animals \((6.63 \pm 0.71)\). However, sCR1\{desLHR-A\}sLe\(^x\) binding to lungs of

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**Table II. Protective effects of sCR1 (desLHR-A) and sCR1 (desLHR-A) sLe\(^x\) in CVF model**

<table>
<thead>
<tr>
<th>Material Infused</th>
<th>Dose (mg)</th>
<th>Permeability</th>
<th>MPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCR1 (desLHR-A)</td>
<td>0.30</td>
<td>0.61 ± 0.03 (NS)*</td>
<td>0.48 ± 0.03 (NS)*</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.44 ± 0.02 (&lt;0.001)*</td>
<td>0.38 ± 0.02 (0.002)*</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>0.38 ± 0.02 (&lt;0.001)*</td>
<td>0.33 ± 0.02 (&lt;0.001)*</td>
</tr>
<tr>
<td>sCR1 (desLHR-A)sLe(^x)</td>
<td>0.30</td>
<td>0.54 ± 0.02 (NS)**</td>
<td>0.43 ± 0.02 (NS)**</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.38 ± 0.02 (NS)**</td>
<td>0.34 ± 0.02 (NS)**</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>0.30 ± 0.02 (0.03)**</td>
<td>0.25 ± 0.01 (0.01)**</td>
</tr>
</tbody>
</table>

* Permeability values for positive and negative control groups were 0.64 ± 0.02 and 0.15 ± 0.01, respectively. The MPO values for positive and negative control groups were 0.85 ± 0.02 and 0.11 ± 0.02, respectively. For each group \(n = 5\).

*\(p\) values when compared to positive control group that was otherwise untreated.

**\(p\) values when similar doses for the two groups were compared at similar doses. NS, not significant \((p > 0.05)\).
the lines between groups represent

The MPO value for the negative control group was 0.27 ± 0.04. Numbers connecting the lines between groups represent *p* values. For each group, *n* = 5.

### Table V. Binding of sCR1[desLHR-A] and sCR1[desLHR-A]sLe⁶ to rat lungs after IgG immune complex deposition

<table>
<thead>
<tr>
<th>Group</th>
<th>sCR1[desLHR-A]</th>
<th>sCR1[desLHR-A]sLe⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.60 ± 0.50</td>
<td>11.1 ± 1.0</td>
</tr>
<tr>
<td>sCR1[desLHR-A]</td>
<td>5.40 ± 0.30</td>
<td>25.4 ± 1.0</td>
</tr>
<tr>
<td>sCR1[desLHR-A]sLe⁶</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Details are described in text. Binding was assessed 4 h after initiation of the inflammatory response. Statistical analysis (positive) is indicated by the numbers between lines connecting the groups compared. For each group, *n* = 5. Binding was determined using 125I-labeled compounds.

animals infused with CVF and MOPC-21 was much higher (13.7 ± 0.76 μg). In animals infused with saline in the presence of anti-P-selectin (PB1.3), the binding of both sCR1[desLHR-A] and sCR1[desLHR-A]sLe⁶ was relatively low and similar (5.41 ± 0.29 and 7.93 ± 0.29, respectively; *p*, NS). Binding of sCR1[desLHR-A] in the presence of PB1.3 was similar in lungs of animals infused with saline (5.41 ± 0.29) or CVF (7.93 ± 0.74) (*p*, NS). With sCR1[desLHR-A]sLe⁶, binding in saline-infused animals also treated with PB1.3 was not statistically different (7.91 ± 0.29) (*p*, NS). However, in CVF-treated rats in the presence of PB1.3, the binding of sCR1[desLHR-A]sLe⁶ fell to 10.1 ± 0.54 μg in CVF-infused animals, a significant drop (*p* = 0.04) from the 13.7 ± 0.76 value in the CVF-infused animals also receiving MOPC-21. These data indicate that, in CVF-treated rats, there is increased specific binding of sCR1[desLHR-A]sLe⁶ to the lung vasculature when compared with the undecorated form of this molecule and that this binding can be significantly diminished by the presence of anti-P-selectin (PB1.3).

**Protective effects of sCR1[desLHR-A] and sCR1[desLHR-A]sLe⁶ in injury caused by IgG immune complexes**

The complement inhibitors sCR1[desLHR-A] and sCR1[desLHR-A]sLe⁶ were evaluated in the L-selectin-, P-selectin-, and E-selectin-dependent model of acute vascular injury caused by the intrapulmonary deposition of IgG immune complexes. These inhibitors were infused at a dose of 4.5 mg. Permeability indices and MPO activity were assessed (as described above). The results are summarized in Table IV. Treatment with sCR1[desLHR-A] reduced the permeability index and MPO values by 45% (*p* = 0.04) and 50%, (*p* < 0.001), respectively, while treatment with sCR1[desLHR-A]sLe⁶ reduced these values by 63% (*p* < 0.002) and 71%, (*p* = 0.005), respectively. For both permeability indices and MPO values, the differences in effects of sCR1[desLHR-A] as compared with those of sCR1[desLHR-A]sLe⁶ were statistically significantly different, with the latter being more protective. Thus, in both models of acute lung injury, sCR1[desLHR-A]sLe⁶ at the 4.5-mg dose demonstrated greater protective effects than sCR1[desLHR-A].

**Lung vascular binding of sCR1[desLHR-A] and sCR1[desLHR-A]sLe⁶ after intrapulmonary deposition of IgG immune complexes**

We evaluated the binding of 125I-sCR1[desLHR-A] and 125I-sCR1[desLHR-A]sLe⁶ 3 h and 45 min after initiation of IgG immune complex-induced lung injury. The animals were infused with 4.5 mg of sCR1[desLHR-A] or sCR1[desLHR-A]sLe⁶, together with 125I-labeled compounds, to provide 800,000 cpm per animal. Fifteen minutes later, the animals were sacrificed. Binding data are shown in Table V. Binding values for sCR1[desLHR-A] in lungs of negative (no IgG immune complexes) and positive (IgG immune complexes) control groups were 5.60 ± 0.50 and 5.40 ± 0.30 μg, respectively. In the negative control groups, the binding of sCR1[desLHR-A]sLe⁶, as compared with binding of sCR1[desLHR-A], was doubled in the negative control group,
In the positive control group, binding of sCR1[desLHR-A]sLex rose to 25.4 ± 1.0 μg, more than 2-fold above the level in the negative control group, and nearly 5-fold when compared with amounts of sCR1[desLHR-A] bound in negative or positive control lungs. Accordingly, the binding of sCR1[desLHR-A] and sCR1[desLHR-A]sLex to the lung vasculature correlated with the protective effects of these compounds (Table IV).

In vitro binding of 125I-sCR1[desLHR-A] and 125I-sCR1[desLHR-A]sLex to nonactivated and activated endothelial cells

The binding of 125I-sCR1[desLHR-A] and 125I-sCR1[desLHR-A]sLex to unstimulated and TNF-α-stimulated (4 h) HUVEC was assessed. When unstimulated endothelial cells were incubated with 125I-sCR1[desLHR-A], very little binding occurred (Fig. 2A). This low level of binding was unaffected by the presence of anti-E-selectin (CL-3), anti-P-selectin (PB1.3), or a class-matched irrelevant IgG1 (MOPC-21). In TNF-α-stimulated HUVEC, there was, likewise, no significant increase in binding of sCR1[desLHR-A] (Fig. 2B). In contrast, the binding patterns of sCR1[desLHR-A]sLex were quite different. Although binding of sCR1[desLHR-A]sLex to unstimulated HUVECs (Fig. 2C) was about 2.5-fold higher than the binding of sCR1[desLHR-A] to unstimulated cells (Fig. 2A), there was a significantly greater increase in the binding of sCR1[desLHR-A]sLex to the TNF-α-stimulated HUVECs in the absence of Ab (Fig. 2D). This binding was greatly reduced in the presence of anti-E-selectin (CL-3) but not in the presence of either anti-P-selectin or the subclass-matched irrelevant mouse IgG1 (MOPC-21). Thus, in vitro binding of sCR1[desLHR-A]sLex was increased in TNF-α-stimulated HUVECs in a manner that proved to be E-selectin dependent.

Discussion

Adhesiveness of neutrophils to the endothelium is a key event in the early inflammatory response. Rapid endothelial expression of P-selectin appears to facilitate early adhesive interactions involving counterreceptors on the neutrophil, such as P-selectin glycoprotein ligand-1 (PSGL-1) and, to a lesser extent, E-selectin ligand-1 (ESL-1). The current report describes a novel strategy that takes advantage of the known interactions between certain selectins (chiefly L- and P-selectins) and sialated, fucosylated oligosaccharides, especially sLex (13, 14). A process that permits incorporation of the sLe^x moiety into natural N-linked oligosaccharides has been developed. This has the advantage of sLe^x tetrasaccharide being attached to large, naturally occurring oligosaccharides. In addition to the possibility that sLex-bearing glycoproteins may inhibit the binding of selectins to their counterreceptors, these modified proteins may also become localized in vivo to sites on the activated endothelium because of their content of sLex^x, hereby allowing complement inhibition to be focused at the site of the activated endothelium. In addition or alternatively, this binding might compete with the ability of neutrophils to bind to their counterreceptors (selectins) on endothelial cells.

Ischemic injury of the myocardium (32), of the hind limbs (33), and of lung (34) is in every case associated with participation of selectins. Blocking Ab to human E-selectin (CL-3) and to P-selectin (PB1.3) were added at concentrations of 5 μg/ml. MOPC-21 was a subclass-matched control. Stimulation of HUVEC was accomplished by addition of 50 ng/ml human TNF-α at 37°C for 4 h before washing and further additions. The binding of 125I-sCR1[desLHR-A]sLex to unstimulated HUVEC (C) and TNF-α-stimulated HUVEC (D) is shown.
In addition, the binding of sCR1sLex to either endothelial cells has been linked to up-regulation of endothelial P-selectin activation, and, therefore, in inhibiting complement activation, than sCR1 (which does not bind) in preventing local complement activation of neutrophils within the lung.

This increased efficacy of sCR1sLe^a as compared with sCR1 in protecting against CVF-induced lung injury seems to be related to the ability of sCR1sLe^a to bind to the activated endothelium. By immunohistochemical methods, we were able to demonstrate that sCR1sLe^a, but not sCR1, was bound to the lung vascular endothelium following systemic activation of complement (Fig. 1). This may imply that endothelial-bound sCR1sLe^a is more effective than sCR1 (which does not bind) in preventing local complement activation, and, therefore, in inhibiting complement activation, which has been linked to up-regulation of endothelial P-selectin (31). In addition, the binding of sCR1sLe^a to either endothelial cells or to neutrophils might interfere with the binding interactions between these cells (via endothelial P-selectin and/or neutrophil L-selectin, or both), resulting in diminished accumulation of neutrophils within the lung.

The presence of sLe^a on sCR1, which results in a more protective molecule, appears not to be associated with altered pharmacokinetics, or due to sCR1sLe^a having superior complement inhibitory activities (see above). Further, although platelets in addition to endothelial cells can be stimulated by complement activation products to express P-selectin (35–37), platelets appear unlikely to be the source of P-selectin in the CVF model of lung injury, since in this model platelet depletion did not reduce the intensity of lung injury or lung vascular expression of P-selectin (31). In the CVF model, we also observed increased efficacy of sCR1[desLHR-A]sLe^a relative to sCR1[desLHR-A] based on reduced lung permeability and diminished content of MPO. That correlated with greater binding of sCR1[desLHR-A]sLe^a to the lung vasculature when compared with sCR1[desLHR-A] (Table III). This binding was P-selectin-dependent and was determined by the use of anti-P-selectin. Therefore, the enhanced protective effects of sCR1[desLHR-A]sLe^a are consistent with the interpretation that sCR1[desLHR-A]sLe^a has greater binding to the activated endothelium. As expected, deletion of domain A in sCR1 (sCR1[desLHR-A]) as compared with intact sCR1 (19 vs 37; 38 vs 46 nM) had little effect on its inhibitory activity in the alternative pathway, while, at the same time, causing nearly 50-fold reduction in inhibition of the classical pathway (see above). There seems little question that, even in the CVF model of lung injury, sCR1[desLHR-A] was less effective than the sLe^-decorated forms of sCR1 and sCR1[desLHR-A]. This correlated with the enhanced binding activities of these compounds to the lung vasculature (Fig. 1 and Table III).

Using the IgG immune complex model of lung injury, which is L-selectin-, P-selectin-, and E-selectin-dependent, the ability of sLe^-decorated and undecorated forms of sCR1[desLHR-A] was evaluated both for protective effects as well as binding to the lung vasculature. Undeniably, both inhibitors showed protective effects, with the decorated versions being more effective (Table IV). This also correlated with the higher binding of the sLe^-decorated form to the lung vasculature (Table V). What is curious is why sCR1[desLHR-A] would have any protective effects in the IgG immune complex model (which would be assumed to be predominately engaging the classical complement pathway). SCR1[desLHR-A], when compared with sCR1, had somewhat reduced blocking activity for the alternative pathway (IC50 values of 37 ± 6.2 nM vs 19 ± 6.6 nM, respectively). If the developing IgG immune complex response at some point were to engage the alternative pathway due to generation of C3b, this could explain why sCR1[desLHR-A] had protective effects (albeit diminished). Such a possibility is supported by published data (38–41). When sCR1 and sCR1[desLHR-A] were evaluated in vitro for their complement inhibiting activities, the IC50 values for inhibition of the classical and alternative pathways were 0.21 nM and 19 nM for sCR1 and 58 nM and 37 nM for sCR1[desLHR-A] (above and Footnote 4). Therefore, if present in sufficient concentrations, sCR1[desLHR-A] would contain the ability to block activation of the classical pathway. Intravenous infusion of decorated or undecorated sCR1[desLHR-A] (at 15 mg/kg body weight) would yield a plasma concentration in the range of 2 μM, well above the IC50 values for inhibition of the classical pathway. As to why the sLe^-decorated form of sCR1[desLHR-A] was a more effective inhibitor of injury in the IgG immune complex model of lung injury than was the undecorated form, recent observations that this model is both P-selectin and L-selectin dependent (and also E-selectin dependent (see above)) would be consistent with the ability of sLe^- containing oligosaccharides to interact with all three selectins. As to why sCR1[desLHR-A]sLe^a but not the sLe^-undecorated form binds to TNF-α-stimulated HUVECs in an E-selectin manner, these data are consistent with published evidence that E-selectin recognizes the monomeric sLe^- moiety and that the strength of binding between E-selectin and monomeric sLe^- is comparable to the binding interactions between P- or E-selectin and monomeric sLe^- (42–47). Interactions between P-selectin and its primary counterreceptor, PSGL-1, depend on sLe^- decoration of PSGL-1. It is believed that this interaction is higher affinity than that between P-selectin and monomeric sLe^- because of 1) tyrosine sulfation on PSGL-1 that contributes to binding affinity, 2) the multivalent nature of the sLe^- moieties on O-linked glycans present on PSGL-1, and 3) the overall tertiary conformation assumed by these glycans. Similar considerations also apply to interactions between L-selectin on rolling neutrophils and PSGL-1 displayed by adherent neutrophils, which is the major counterreceptor recognized by L-selectin that is operative in neutrophil recruitment. The nature of bona fide neutrophil E-selectin ligands is less clear. There is evidence that mono- and polysaccharidic glycoconjugates are physiological ligands, and there is evidence that PSGL-1 is also a ligand.

The strategy to develop complement inhibitors that can be “targeted” to the selectin-expressing activated endothelium is attractive, since this should provide a way to achieve localization of a complement inhibitor along surfaces of the activated endothelium. Collectively, our data suggest that decoration of sCR1 or sCR1[desLHR-A] with sLe^- enhances their binding to the selectin-expressing vascular endothelium and, in turn, enhances protection...
against neutrophil-mediated injury. Whether the enhanced protective effects of sCR1Le and sCR1[desLHR-H]-Le are due to their increased concentration at sites of the vascular endothelium (thus more effectively inhibiting local complement activation) or the sLe^-decorated compounds complete with selectin-dependent binding interactions of neutrophils to the activated endothelium remains to be determined. sCR1Le and sCR1[desLHR-H]-Le are clearly more effective antiinflammatory agents when compared with the forms lacking sLe^- This may suggest a novel strategy for development of antiinflammatory compounds.

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References