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


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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 selectin-dependent 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 nonidiotypic 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)\textsuperscript{3} (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 (SCRs), 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 for the most common human allotype of CR1. sCR1 was prepared by deleting the cytoplasmic and transmembrane domains while retaining LHR-A, -B, -C, and -D (10, 11). This recombinant molecule blocked the assembly of enzymes (convertases) responsible for cleavage of C3 and C5 and subsequent activation of the complement system and served as a cofactor in the proteolysis of C3b and C4b by Factor I. sCR1 has been shown to inhibit both classical and alternative pathways of complement activation (10, 11). An additional soluble version of sCR1 has been constructed by deleting LHR-A (as well as the cytoplasmic and transmembrane domains). The resulting recombinant molecule, consisting of LHR-B, -C, and -D, has been designated sCR1[desLHR-A] (12). Because sCR1[desLHR-A] lost the C4b binding site contained in LHR-A, it should be a relatively selective (but not absolute) inhibitor 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 sialylated, fucosylated 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}.
moiety (19). In the current report, we assessed sLe\textsuperscript{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\textsuperscript{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\textsuperscript{x}.

One method to obtain proteins with the sLe\textsuperscript{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\textsuperscript{x} motif within natural N-linked oligosaccharides. This strategy was applied for production of modified proteins, sCR1sLe\textsuperscript{x} and sCR1(desLHR-A)sLex. These proteins have been shown to possess sLe\textsuperscript{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\textsuperscript{x} and sCR1(desLHR-A)sLex 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\textsuperscript{x} tetrasaccharide have been shown to be protective (7, 27, 28). The current studies indicate that sLe\textsuperscript{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)sLex were labeled with 125I [New England Nuclear, Boston, MA] using lactoperoxidase techniques. sCR1 and sCR1(desLHR-A) produced by DUKX B11 possessed no sLe\textsuperscript{x} on their N-linked oligosaccharides (48) (M. D. Picard et al., manuscript in preparation). For sCR1sLe\textsuperscript{x} production, the expression plasmid coding for sCR1 was used (11). The plasmid pTCSLDHFR\textsuperscript{B*} coding for a mutant mouse dihydrofolate reductase with an abnormally low affinity for methotrexate (29), was applied for production of modified proteins, sCR1sLex and sCR1(desLHR-A)sLe\textsuperscript{x}. These proteins have been shown to possess sCR1(desLHR-A)sLex production, the expression plasmid coding for sCR1 was used (11). The plasmid pTCSLDHFR\textsuperscript{B*} coding for a mutant mouse dihydrofolate reductase with an abnormally low affinity for methotrexate (29), was applied for production of modified proteins, sCR1sLex and sCR1(desLHR-A)sLe\textsuperscript{x}.

Animal models and in vivo binding assays

CVF was isolated from Naja naja venom by a combination of ion exchange and gel filtration units (5). For in vivo binding studies, animals were injected i.v. as a single bolus into young male, specific pathogen-free, Long-Evans rats (300 to 350 g). For binding studies, 0.5 mCi of 125I-sCR1(desLHR-A) or 125I-sCR1(desLHR-A)sLe\textsuperscript{x} together with unlabeled forms (1.5 mg) of the same compounds was infused i.v. just before i.v. infusion of either sterile saline or CVF. Some animals also received an i.v. infusion of 200 μg of either PB1.3 (IgG1 anti-P-selectin) or MOPC-21 (a subclass-matched Ab) just before infusion of CVF together with 125I-sCR1(desLHR-A) or 125I-sCR1(desLHR-A)sLe\textsuperscript{x}. PB1.3 is a monoclonal mouse IgG1 with reactivity to human P-selectin and cross-reactivity to rat P-selectin (30). For lung binding studies, animals were killed 20 min after i.v. infusion of CVF. This is the time at which lung vascular P-selectin peaks in vivo (31). For measurement of injury parameters, 0.5 mCi of 125I-BSA was injected i.v. just before infusion of CVF. Unless otherwise indicated, animals were sacrificed 30 min later, and the pulmonary arterial circulation was flushed with 10 ml of sterile saline to remove residual blood (and blood-associated 125I-BSA). The amount of radioactivity in the lungs was compared with that present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice. Permeability and hemorrhage indices were calculated, as described elsewhere (30). Briefly, the permeability index was calculated by the ratio of radioactivity (125I-BSA) present in saline-perfused lungs 30 min after i.v. infusion of CVF to the amount of radioactivity present in 1.0 ml of blood.

Lung injury was also induced with a rabbit polyclonal IgG rich in Ab to BSA (Organon Teknika, West Chester, PA) as previously described (23–26). As indicated above, lung injury in this model requires engagement of all three selectins. Briefly, 2.5 mg anti-BSA in 300 μl was instilled into rat lungs via a tracheal cannula. In the positive controls, this was followed by an i.v. injection of 10 mg of BSA together with trace amounts of 125I-BSA. In the negative controls, the i.v. infusion of 10 mg BSA was omitted. For binding studies in the IgG immune complex model, similar amounts of nonlabeled and 125I-labeled sCR1(desLHR-A) or sCR1(desLHR-A)sLe\textsuperscript{x} were administered in 4.5 mg of unlabeled sCR1 derivatives were infused i.v. 20 min before sacrifice, at 4 h. This is when up-regulation of P- and E-selectins are maximally in this model of injury (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\textsuperscript{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\textsuperscript{x} versions) compared with those expressed by DUKX-B11 cells (non-sLe\textsuperscript{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. As a result of the results of a number of experiments, the concentrations of sCR1sLe\textsuperscript{x} required to yield half-maximal lysis of sensitized sheep erythrocytes was somewhat higher (IC\textsubscript{50} = 0.27 ± 0.082 nM, n = 31) than that required for sCR1 (IC\textsubscript{50} = 0.21 ± 0.060 nM, n = 65). This suggests that sCR1sLe\textsuperscript{x} is a slightly less effective inhibitor of classical complement activation. Similarly, in the assay of alternative pathway lysis of guinea pig erythrocytes, sCR1sLe\textsuperscript{x} appeared somewhat less effective (IC\textsubscript{50} = 38 ± 16 nM, n = 37) than sCR1 (IC\textsubscript{50} = 19 ± 6.6 nM, n = 10). Analogous results were obtained for the two versions of sCR1(desLHR-A). In the classical pathway assay, sCR1(desLHR-A)sLe\textsuperscript{x} (IC\textsubscript{50} = 140 ± 32 nM, n = 3) was somewhat less effective than sCR1(desLHR-A) (IC\textsubscript{50} = 58 ± 38 nM, n = 4) but much less effective than either version of sCR1. In the alternative pathway assay, sCR1(desLHR-A)sLe\textsuperscript{x} (IC\textsubscript{50} = 46 ± 9.1 nM, n = 4) was again somewhat less effective than sCR1(desLHR-A) (IC\textsubscript{50} = 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\textsuperscript{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\textsuperscript{x} (10 ng/ml) together with 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 lyed 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 unlabeled sCR1[desLHR-A] or sCR1[desLHR-A][sLex].

**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 sCR1sLex**

The relative efficacy of sCR1 and sCR1sLex 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 sCR1sLex, 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, sCR1sLex 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 sCR1sLex 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, sCR1sLex is a more effective inhibitor than is sCR1.

The observed differences in the efficacy of sCR1 and sCR1sLex could not be explained by differences in the pharmacokinetic or complement-inhibiting properties of the compounds. The pharmacokinetics of sCR1 and sCR1sLex 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 sCR1sLex. Plasma samples (obtained at 0, 3, 10, 30, and 60 min during the first hour) were quantitated for sCR1 and sCR1sLex 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 sCR1sLex cannot be explained by differences in pharmacokinetics. With respect to complement-inhibiting properties of sCR1 and sCR1sLex, similar concentrations of sCR1 and sCR1sLex showed similar inhibition in human serum complement-mediated lysis of Ab-sensitized sheep erythrocytes (see above).

**Immunostaining of sCR1 and sCR1sLex in rat lung after CVF infusion**

Using immunostaining to detect binding of sCR1 and sCR1sLex 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 sCR1sLex, 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 sCR1sLex) 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 sCR1sLex and CVF resulted in obvious evidence of binding of sCR1sLex to lung interstitial capillaries and venules (Fig. 1, B and D). These data provide direct evidence for

**Table I. Protective effects of sCR1 and sCR1sLex after CVF infusion**

<table>
<thead>
<tr>
<th>Material Infused</th>
<th>Dose (mg)</th>
<th>Permeability</th>
<th>MPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCR1</td>
<td>0.30</td>
<td>1.05 ± 0.05 (NS)*</td>
<td>1.11 ± 0.05 (0.04)*</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.90 ± 0.02 (0.004)*</td>
<td>0.95 ± 0.06 (0.002)*</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>0.81 ± 0.05 (0.02)*</td>
<td>0.88 ± 0.05 (0.03)*</td>
</tr>
<tr>
<td>sCR1sLex</td>
<td>0.30</td>
<td>0.60 ± 0.06 (&lt;0.001)**</td>
<td>0.95 ± 0.03 (0.003)**</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.462 ± 0.03 (&lt;0.001)**</td>
<td>0.62 ± 0.03 (0.003)**</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>0.38 ± 0.03 (&lt;0.001)**</td>
<td>0.43 ± 0.02 (&lt;0.001)**</td>
</tr>
</tbody>
</table>

* Permeability values for positive and negative control groups were 1.28 ± 0.11 and 0.15 ± 0.01, respectively. The MPO values for positive and negative control groups were 1.29 ± 0.05 and 0.27 ± 0.02, respectively. For each group n = 5. 
**p values when compared with positive control group that was otherwise untreated.

p Values when the companion groups in the same columns were compared at similar doses of sCR1 or sCR1sLex.
the binding of sCR1Le\textsuperscript{e}, 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\textsuperscript{e} after CVF infusion**

We evaluated the protective effects of sCR1[desLHR-A] and sCR1[desLHR-A]sLe\textsuperscript{e} 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\textsuperscript{e}, 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\textsuperscript{e} 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\textsuperscript{e} achieved statistical significance ($p = 0.01$) when compared with sCR1[desLHR-A]. Thus, although the sLe\textsuperscript{e}-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\textsuperscript{e} 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\textsuperscript{e} in the CVF model of lung injury. The results are shown in Table III. The binding of sCR1[desLHR-A] and sCR1[desLHR-A]sLe\textsuperscript{e} 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 lungs of sCR1[desLHR-A]sLe\textsuperscript{e} 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 ($6.73 \pm 0.71$). However, sCR1[desLHR-A]sLe\textsuperscript{e} binding to lungs of

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**Table II. Protective effects of sCR1 (desLHR-A) and sCR1 (desLHR-A) sLe\textsuperscript{e} 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\textsuperscript{e}</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 $n = 5$.

*Statistical significance when compared to positive control group that was otherwise untreated.

**Statistical significance when similar doses for the two groups were compared at similar doses. NS, not significant ($p > 0.05$).**
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]sLex 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]sLe, 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]sLe reduced significantly (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]sLe 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]sLex in injury caused by IgG immune complexes

The complement inhibitors sCR1[desLHR-A] and sCR1[desLHR-A]sLex 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]sLe 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]sLex were statistically significantly different, with the latter being more protective. Thus, in both models of acute lung injury, sCR1[desLHR-A]sLex 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]sLex after intrapulmonary deposition of IgG immune complexes

We evaluated the binding of 125I-sCR1[desLHR-A] and 125I-sCR1[desLHR-A]sLex 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]sLe, 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]sLe, as compared with binding of sCR1[desLHR-A], was doubled in the negative control group, to

Table III. Binding of sCR1[desLHR-A] and sCR1[desLHR-A]sLe to rat lungs after infusion of saline or CVF

<table>
<thead>
<tr>
<th>Material Infused</th>
<th>Ab (200 μg)</th>
<th>Amount (μg) Bounda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sCR1[desLHR-A]</td>
<td>sCR1[desLHR-A]sLex</td>
</tr>
<tr>
<td>Saline MOPC-21</td>
<td>5.78 ± 0.53</td>
<td>6.34 ± 0.31</td>
</tr>
<tr>
<td>CVF MOPC-21</td>
<td>6.63 ± 0.71</td>
<td>13.7 ± 0.76</td>
</tr>
<tr>
<td>Saline PB1.3</td>
<td>5.41 ± 0.29</td>
<td>7.91 ± 0.29</td>
</tr>
<tr>
<td>CVF PB1.3</td>
<td>7.93 ± 0.74</td>
<td>10.1 ± 0.54</td>
</tr>
</tbody>
</table>

a Statistical analysis (p value) is indicated by the numbers between lines connecting the groups compared. NS, p > 0.05.

Table IV. Comparison of protective effects of sCR1[desLHR-A] and sCR1[desLHR-A]sLe in rat lungs after IgG immune complex deposition

<table>
<thead>
<tr>
<th>Treatment (4.5 mg)</th>
<th>Parameter of Lung Injurya</th>
<th>Permeability</th>
<th>MPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.54 ± 0.1</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>sCR1[desLHR-A]</td>
<td></td>
<td>0.36 ± 0.1</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>sCR1[desLHR-A]sLex</td>
<td></td>
<td>0.29 ± 0.2</td>
<td>0.47 ± 0.02</td>
</tr>
</tbody>
</table>

a The mean permeability value for the negative control group was 0.14 ± 0.02. 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.
11.1 ± 1.0 μg, perhaps due to vascular perturbations following airway instillation of anti-BSA (in the absence of i.v. infused BSA). 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]sLe haute 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]sLe haute to nonactivated and activated endothelial cells

The binding of 125I-sCR1[desLHR-A] and 125I-sCR1[desLHR-A]sLe haute 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]sLe haute were quite different. Although binding of sCR1[desLHR-A]sLe haute 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]sLe haute to the TNF-α-stimulated HUVECs (Fig. 2D) as compared to 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]sLe haute 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 (EEL-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 sLe haute (13, 14). A process that permits incorporation of the sLe haute moiety into natural N-linked oligosaccharides has been developed. This has the advantage of sLe haute tetrasaccharide being attached to large, naturally occurring oligosaccharides. In addition to the possibility that sLe haute-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 sLe haute, thereby 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]sLe haute to unstimulated HUVEC (C) and TNF-α-stimulated HUVEC (D) is shown.

FIGURE 2. The binding of 125I-sCR1[desLHR-A] to unstimulated HUVECs (A) and to TNF-α-stimulated HUVEC (B). When used, blocking Abs 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]sLe haute to unstimulated HUVEC (C) and TNF-α-stimulated HUVEC (D) is shown.
In addition, the binding of sCR1sLex to either endothelial which 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 trophils within the lung.

L-selectin, or both), resulting in diminished accumulation of neutrophil between these cells (via endothelial P-selectin and/or neutrophil sulfation on PSGL-1 that contributes to binding affinity, 2) the multivalent nature of the sLeα 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 and its primary counterreceptor, PSGL-1, depend on sLeα-decoration of PSGL-1. It is believed that this interaction is higher affinity than that between P-selectin and monomeric sLeα because of 1) tyrosine sulfation on PSGL-1 that contributes to binding affinity, 2) the multivalent nature of the sLeα 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 lectin that is operative in neutrophil recruitment. The nature of bona fide neutrophil E-selectin ligands is less clear. There is evidence that

The increased efficacy of sCR1sLeα as compared with sCR1 in protecting against CVF-induced lung injury seems to be related to the ability of sCR1sLeα to bind to the activated endothelium. By immunohistochemical methods, we were able to demonstrate that sCR1sLeα, but not sCR1, was bound to the lung vascular endothelium following systemic activation of complement (Fig. 1). This may imply that endothelial-bound sCR1sLeα 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 sCR1sLeα 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 sLeα on sCR1, which results in a more protective molecule, appears not to be associated with altered pharmacokinetics, or due to sCR1sLeα 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]sLeα relative to sCR1[desLHR-A] based on reduced lung permeability and diminished content of MPO. That correlated with greater binding of sCR1[desLHR-A]sLeα 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]sLeα are consistent with the interpretation that sCR1[desLHR-A]sLeα 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 sLeα-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 sLeα-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 sLeα-decorated form to the lung vascular (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 sLeα-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 sLeα containing oligosaccharides to interact with all three selectins. As to why sCR1[desLHR-A]sLeα but not the sLeα-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 sLeα moiety and that the strength of binding between E-selectin and monomeric sLeα is comparable to the binding interactions between P- or L-selectin and monomeric sLeα (42–47). Interactions between P-selectin and its primary counterreceptor, PSGL-1, depend on sLeα-decoration of PSGL-1. It is believed that this interaction is higher affinity than that between P-selectin and monomeric sLeα because of 1) tyrosine sulfation on PSGL-1 that contributes to binding affinity, 2) the multivalent nature of the sLeα 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 polyfucosylated glycolipids 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 sLeα enhances their binding to the selectin-expressing vascular endothelium and, in turn, enhances protection...
against neutrophil-mediated injury. Whether the enhanced protective effects of sCR1sLex and sCR1[desLHR-A]sLex are due to their increased concentration at sites of the vascular endothelium (thus more effectively inhibiting local complement activation) or the sLex-decorated compounds complete with selectin-dependent binding interactions of neutrophils to the activated endothelium remains to be determined. sCR1sLex and sCR1[desLHR-A]sLex are clearly more effective antiinflammatory agents when compared with the forms lacking sLeα. This may suggest a novel strategy for development of antiinflammatory compounds.

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References


