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


J Immunol 1999; 162:4952-4959; 
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Endothelial Targeting and Enhanced Antiinflammatory Effects of Complement Inhibitors Possessing Sialyl Lewis\(^x\) Moieties\(^1\)

Michael S. Mulligan,* Roscoe L. Warner,† Charles W. Rittershaus,§ Lawrence J. Thomas, § Una S. Ryan, § Kimberly E. Foreman, † Larry D. Crouch, ‡ Gerd O. Till, † and Peter A. Ward\(^2\)†

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\(^x\) (sLe\(^x\)), as N-linked oligosaccharide adducts. These modified proteins, sCR1sLe\(^x\) and sCR1[desLHR-A]sLe\(^x\), 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\(^x\) and sCR1[desLHR-A]sLe\(^x\) occurred in a P-selectin-dependent manner, in contrast to the absence of any increased albumin extravasation in lung when compared with the non-sLe\(^x\)-decorated forms. In this model, increased lung vascular binding of sCR1sLe\(^x\) and sCR1[desLHR-A]sLe\(^x\) 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\(^x\) 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\(^x\) as compared with the non-sLe\(^x\)-decorated form. In TNF-\(\alpha\)-activated HUVEC, substantial in vitro binding occurred with sCR1[desLHR-A]sLe\(^x\) (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\(^x\)-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 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 Lewis\(^x\) 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\(^x\)
moiety (19). In the current report, we assessed SLexdecorated 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 antiinflammatory agents. Second, the presence of the SLex 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 SLex.

One method to obtain proteins with the SLex 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 SLex motif within natural N-linked oligosaccharides. This strategy was applied for production of modified proteins, sCR1SLex and sCR1[desLHR-A]SLex. These proteins have been shown to possess SLex 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 sCR1SLex 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 SLex tetrasaccharide have been shown to be protective (7, 27, 28). The current studies indicate that SLex-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 recombinant was produced in the Chinese hamster ovary cell line DUKK B11 and purified as previously described (11). sCR1[desLHR-A] was produced in the DUKK 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 DUKK B11 possessed no SLex on their N-linked oligosaccharides (48) (M. D. Picard, et al., manuscript in preparation). For sCR1SLex production, the expression plasmid coding for sCR1 was used (11). The plasmid pTCSLDHFR, coding for a mutant mouse fucosyltransferase, was transfected into the baby hamster fibroblast line (11). The plasmid pTCSLDHFR* was transfected into the LEC11 cell line (20), which expresses the α2,8-N-acetyllactosamine α1-3 linked fucose during the course of normal oligosaccharide synthesis (20, 21). This would allow production of the SLex motif within natural N-linked oligosaccharides. This strategy was applied for production of modified proteins, sCR1SLex and sCR1[desLHR-A]SLex. These proteins have been shown to possess SLex 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 sCR1SLex 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 SLex tetrasaccharide have been shown to be protective (7, 27, 28). The current studies indicate that SLex-decorated versions of sCR1 are more protective in vivo and have the ability to localize to the activated vascular endothelium.

Animal models and in vivo binding assays
CVF was isolated from Naja naja venom by a combination of ion exchange and gel filtration units techniques (5). Fourteen CVF 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]SLex 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]SLex. 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 inhibition 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 salinelicensed 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]SLex together with 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 is 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 SLex 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 (SLex versions) compared with those expressed by DUKK B11 cells (non-SLex 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 to the results of a number of experiments, the IC50 of sCR1SLex required to yield half-maximal lysis of sensitized sheep erythrocytes was somewhat higher (IC50 = 0.27 ± 0.082 nM, n = 31) than that required for sCR1 (IC50 = 0.21 ± 0.060 nM, n = 65). This suggests that sCR1SLex is a slightly less effective inhibitor of classical complement activation. Similarly, in the assay of alternative pathway lysis of guinea pig erythrocytes, sCR1SLex appeared somewhat less effective (IC50 = 38 ± 16 nM, n = 37) than sCR1 (IC50 = 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]SLex (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]SLex (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]SLex 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]SLex (10 nM) together with unlabeled 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
significance was defined as significantly greater protection (4.5 mg, sCR1 reduced permeability indices by 20%, 34%, and controls (no CVF) (Table I, footnotes). At doses of 0.30, 1.5, and in albumin leak and in MPO content when compared with negative Table I. After infusion of CVF, there were 5- to 10-fold increases ability. Animals were sacrificed 30 min later. Results are shown in
4.5 mg per animal. Inhibitors (200 μg) were infused i.v. immediately before i.v. infusion of 4 U of CVF together with 5 μg of 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
the binding of sCR1Le⁺ 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⁺ after CVF infusion**

We evaluated the protective effects of sCR1[desLHR-A] and sCR1[desLHR-A]sLe⁺ 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⁺ 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⁺ 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⁺ achieved statistical significance (p = 0.01) when compared with sCR1[desLHR-A]. Thus, although the sLe⁺-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⁺ to lung vasculature after CVF infusion**

With availability of 125I-versions of the decorated and undecorated sCR1[desLHR-A] compounds, we evaluated the lung vascular binding of 125I-sCR1[desLHR-A] and 125I-sCR1[desLHR-A]sLe⁺ in the presence or absence of 200 μg 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⁺ to the lung vasculature in the presence of circulating MOPC-21 after infusion of either saline was comparable (5.78 ± 0.53 vs 6.34 ± 0.71 μg, respectively; p, NS). The binding to lung of sCR1[desLHR-A]sLe⁺ in the presence of MOPC-21 in saline-infused animals was similar to sCR1[desLHR-A] to the binding after saline infusion (6.34 ± 0.31) and in CVF-infused animals (6.63 ± 0.71). However, sCR1[desLHR-A]sLe⁺ binding to lungs of

Table II. Protective effects of sCR1 (desLHR-A) and sCR1 (desLHR-A) sLe⁺ 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⁺</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).
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]sLex, 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]sLex 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]sLex 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]sLex 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-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]sLex, as compared with binding of sCR1[desLHR-A], was doubled in the negative control group, to Table V. Binding of sCR1[desLHR-A] and sCR1[desLHR-A]sLex to rat lungs after IgG immune complex deposition

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

<table>
<thead>
<tr>
<th>Material Infused</th>
<th>Ab (200 μg)</th>
<th>Amount (μg) Bound</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>NS</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>

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

* Determined using 125I-labeled compounds, as described in the text.

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

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

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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Amount (μg) Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sCR1[desLHR-A]</td>
</tr>
<tr>
<td>Negative controls</td>
<td>5.60 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>IgG immune complex-tre</td>
<td>5.40 ± 0.30</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.
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]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 sLe^X (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 sLe^X-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^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 P-selectin or the use of sLe^X tetrasaccharide has been shown to be protective in several of these models of injury (31, 34). Ischemia-reperfusion injury appears to be associated with up-regulation of endothelial P-selectin, perhaps in part due to complement activation (C5a and/or C5b-9). Accordingly, ischemic models are logical applications for complement inhibitors decorated with sLe^X. Complement inhibitors would interfere with these pathways by reducing generation of C5a and/or
C5b-9, both of which have been shown to induce P-selectin expression on endothelial cells (35–37). In these various models of ischemia-reperfusion injury, neutrophil recruitment seems to be an important event related to full development of injury.

The method by which sCR1sLe^a and sCR1[desLHR-A]sLe^a are produced is described elsewhere. The analysis of oligosaccharides on these molecules confirms that they do indeed possess the sLe^a moiety. The goal of the current report has been to characterize the biological activity and the efficacy of the sLe^a-decorated glycoproteins in two selectin-dependent models of acute lung injury. We first assessed the protective functions of the sLe^a-decorated and undecorated forms of sCR1 and sCR1[desLHR-A] in the P-selectin- and L-selectin-dependent model of acute lung injury, which occurs following systemic activation of complement following i.v. infusion of CVF. In this model, blocking of P-selectin with anti-P-selectin Ab (PB1.3) has been shown to reduce neutrophil accumulation and lung vascular injury, as measured by albumin leak, hemorrhage, and MPO content (see above). In the same model, infusion of L-selectin- or P-selectin-Ig chimeric proteins (but not E-selectin-Ig chimeric protein) protected against full development of lung injury (24). Furthermore, i.v. infusion of penta- or tetrasaccharide sLe^a was protective in this model, these effects being associated with reduced accumulation of neutrophils and development of injury (19).

The 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^a-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^a-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^a-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^a-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^a containing oligosaccharides to interact with all three selectins. As to why sCR1[desLHR-A]sLe^a but not the sLe^a-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^a moiety and that the strength of binding between E-selectin and monomeric sLe^a is comparable to the binding interactions between P- or L-selectin and monomeric sLe^a (42–47). Interactions between P-selectin and its primary counterreceptor, PSGL-1, depend on sLe^a-decoration of PSGL-1. It is believed that this interaction is higher affinity than that between P-selectin and monomeric sLe^a because of 1) tyrosine sulfation on PSGL-1 that contributes to binding affinity, 2) the multivalent nature of the sLe^a 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 sLe^a enhances their binding to the selectin-expressing vascular endothelium and, in turn, enhances protection
against neutrophil-mediated injury. Whether the enhanced protective effects of sCR1\(\text{L}e^\alpha\) and sCR1[desLHR-A]\(\text{L}e^\alpha\) are due to their increased concentration at sites of the vascular endothelium (thus more effectively inhibiting local complement activation) or the \(\text{L}e^\alpha\)-decorated compounds complete with selectin-dependent binding interactions of neutrophils to the activated endothelium remains to be determined. sCR1\(\text{L}e^\alpha\) and sCR1[desLHR-A]\(\text{L}e^\alpha\) are clearly more effective antiinflammatory agents when compared with the forms lacking \(\text{L}e^\alpha\). This may suggest a novel strategy for development of antiinflammatory compounds.

Acknowledgments

We thank Henry C. Marsh, Jr. for insightful input; Robin Kunkel, Susanne Sceseney, and Christopher Honan for technical support; and Beverly Schumann for secretarial assistance.

References