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## Protective effects of oligosaccharides in P-selectin-dependent lung injury

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NEUTROPHIL recruitment into tissues is a multistep process involving sequential engagement of adhesion molecules, including selectins (E,P,L), which are reactive with oligosaccharides, and the family of  $\beta 2$  integrins which are reactive with endothelial intercellular adhesion molecules<sup>1–3</sup>. These processes result in the initial rolling of leukocytes along the endothelial surfaces, followed by the firm attachment of leukocytes to the endothelium. The intravenous infusion of cobra venom factor into rats results in acute lung injury that is neutrophil-dependent, oxygen radical mediated and P-selectin-dependent<sup>4,5</sup>. Here we report that infusion of sialyl-Lewis X, a ligand for P-selectin<sup>6–9</sup>, dramatically reduced lung injury and diminished the tissue accumulation of neutrophils, whereas irrelevant oligosaccharides had no such effects. These results suggest that sialyl-Lewis X carbohydrates may be used as a new strategy for anti-inflammatory therapy.

We used several sialyl-Lewis X (SLX)-related oligosaccharides, the structures of which are shown in Fig. 1. The ability of the SLX reducing sugar (SLX-OH) to reduce neutrophil accumulation and diminish lung injury after infusion of cobra

venom factor (CVF) is shown in Fig. 2. These effects contrast with the lack of protection when the non-fucosylated form of SLX-OH, sialyl-N-acetyllactosamine (SLN-OH), was used (Fig. 2). The positive controls (injected intravenously with CVF in phosphate buffered (pH 7.4) saline, PBS) showed a sixfold increase in lung permeability (Fig. 2a), a fivefold increase in haemorrhage (b) and a fivefold increase in lung myeloperoxidase (MPO) content. When 200  $\mu$ g SLN-OH (a control carbohydrate which does not support P-selectin-mediated adhesion<sup>8</sup>) was infused immediately before injection of CVF, the permeability, haemorrhage and MPO values were unaffected, whereas treatment with SLX-OH reduced the permeability value by 43% ( $P=0.001$ ), haemorrhage by 41% ( $P=0.004$ ) and MPO content by 35% ( $P=0.006$ ).

Dose-response relationships were evaluated with two other SLX analogues with similar potency: SLX-tetrasaccharide (SLX-tetra) and an SLX-pentasaccharide (SLX-penta). Both SLX analogues showed similar potency with maximal inhibition achieved at a dose of 200  $\mu$ g (Fig. 3). Over a dose range of oligosaccharide of 50–500  $\mu$ g, treatment with SLX-penta reduced the permeability values by as much as 67% (Fig. 3a), haemorrhage by as much as 47% (b), and lung MPO content by as much as 49% (c). These data indicate that three different analogues of SLX have significant protective effects against CVF-induced lung injury, that these protective effects are dose-dependent and plateau at 200  $\mu$ g, and that the protective effects correlate with reduced neutrophil content in the lung, as defined by lung content of MPO. Note that the protective effects of the relevant oligosaccharides (this report) quantitatively parallel the protective effects of murine monoclonal anti-P-selectin in the CVF model of lung injury<sup>5</sup>.

As indicated above, infusion of 200  $\mu$ g SLX-pentasaccharide 5 min before injection of CVF reduced permeability and haemorrhage by 67% ( $P<0.01$ ) and 47% ( $P<0.01$ ), respectively. When the intravenous infusion of SLX-penta was delayed until 5 min after infusion of CVF, permeability and haemorrhage (as compared with values in SLN-treated rats) were reduced by 46% ( $P=0.034$ ) and 38% ( $P=0.013$ ), respectively, whereas when the oligosaccharide was infused 15 min after CVF, the permeability and haemorrhage parameters fell by 36% ( $P=0.007$ ) and 25% ( $P=0.014$ ), respectively. Thus the protective effects of SLX-penta in the CVF model of lung injury are time-dependent in relation to the infusion of CVF.

Rats were infused intravenously with PBS, 200  $\mu$ g SLX-penta or its non-fucosylated analogue (SLN-penta) before treatment with CVF and the lungs were prepared for light microscopy 30 min later. Figure 4 shows the expected evidence of intra-alveolar haemorrhage and neutrophil accumulation along endothelial surfaces, whereas in the SLX-penta-treated animals, there was

### Sialyl-Lewis X (SLX)

	Abbreviation	R group
$\text{NeuAc}\alpha 2,3\text{Gal}\beta 1,4$ $\text{Fuc}\beta 1,3$	SLX-OH	OH
	SLX-tetra	$\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$
	SLX-penta	$\beta 1,3\text{Gal}\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$

### Sialyl-N-Acetyllactosamine (SLN)

	Abbreviation	R group
$\text{NeuAc}\alpha 2,3\text{Gal}\beta 1,4\text{GlcNAc-R}$	SLN-OH	OH
	SLN-tetra	$\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$
	SLN-penta	$\beta 1,3\text{Gal}\beta\text{-O}(\text{CH}_2)_5\text{COOCH}_3$

FIG. 1 Synthetic sialyl-Lewis X (SLX) oligosaccharides and non-fucosylated analogues (SLN) used as inhibitors of CVF-induced lung injury. Oligosaccharides were synthesized by a combined chemical and enzymatic approach<sup>13,14</sup> (S.D. and Z.L.Z., unpublished observations).

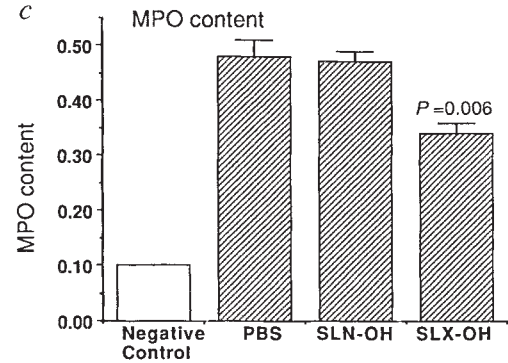
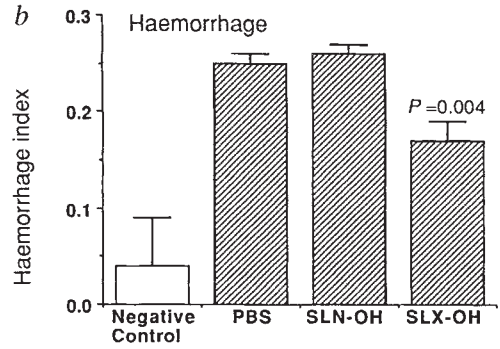
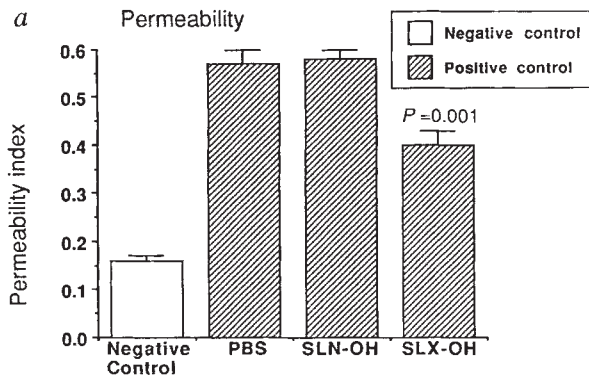


FIG. 2 Lung injury after bolus intravenous infusion of CVF (20 units), as assessed at 30 min by leakage into lung parenchyma of  $^{125}\text{I}$ -BSA (a), extravasation of  $^{51}\text{Cr}$ -rat red blood cells (RBC) (b), and tissue accumulation of neutrophils (MPO content) (c). Negative control rats (300 g male Long Evans) received 0.5 ml PBS intravenously, whereas other groups received 0.25 ml CVF at time 0 preceded 5 min earlier by 0.25 ml PBS, PBS with 200  $\mu\text{g}$  SLX-OH or PBS with 200  $\mu\text{g}$  non-fucosylated form of SLX-OH (SLN-OH). MPO content was determined as described elsewhere<sup>5</sup>. Protection against injury was calculated by subtracting negative control values from each positive control treatment group and then comparing injury in the CVF group with those CVF groups pretreated with oligosaccharide preparations. For each vertical bar,  $n=6$ .

much reduced evidence of these changes.

The concentrations of SLX-OH required to mediate the protective effects in the CVF lung injury model are surprisingly low. It can be calculated that the 200  $\mu\text{g}$  dose of SLX in the rat would be rapidly reduced by dilutional changes to less than 10  $\mu\text{g}$   $\text{ml}^{-1}$  blood, resulting in a blood concentration of  $<1$   $\mu\text{M}$ . The

ability of SLX preparations to show significant protective effects in the CVF model of acute lung injury in the rat provides further evidence that selectin-dependent adhesive interactions between leukocytes and endothelial cells may be appropriate targets of anti-inflammatory interventions based on the use of oligosaccharide analogues of their ligands.

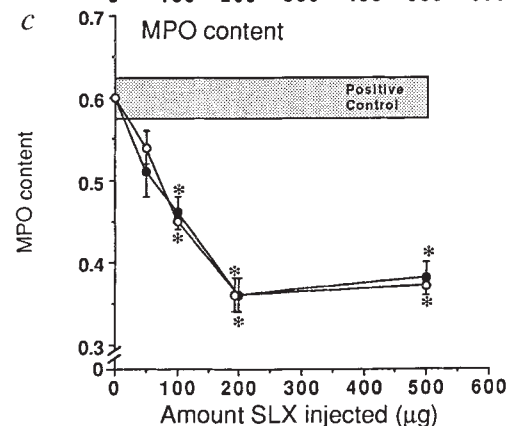
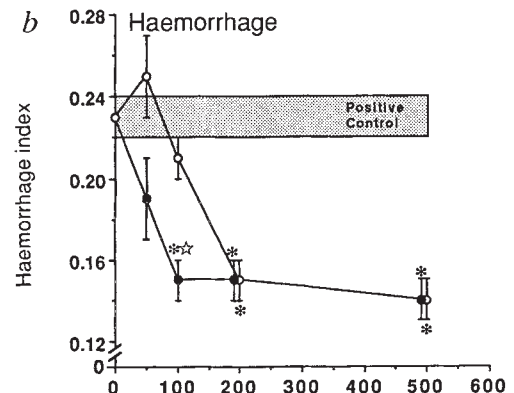
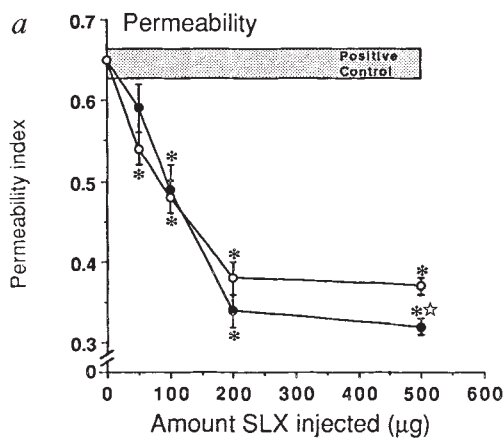


FIG. 3 Dose-response relationships of CVF-infused rats pretreated with varying amounts of tetrasaccharide or pentasaccharide preparations of SLX, used as single intravenous doses (50–500  $\mu\text{g}$ ) in 0.25 min before intravenous infusion of CVF. Lung injury parameters of permeability (a), haemorrhage (b) and MPO content (c) were measured according to details described in Fig. 2. The positive control (animals also receiving 200  $\mu\text{g}$  SLN-OH) reference points (mean  $\pm$  s.e.m.) are indicated by the horizontal area ('positive control'). For each data point,  $n=5$ . ○, SLX-tetra; ●, SLX-penta. \*  $P<0.01$  (a,c),  $<0.05$  (b) when compared with positive controls; †  $P<0.05$  (a), or  $<0.01$  (b) when compared with tetrasaccharide group at same dose level.

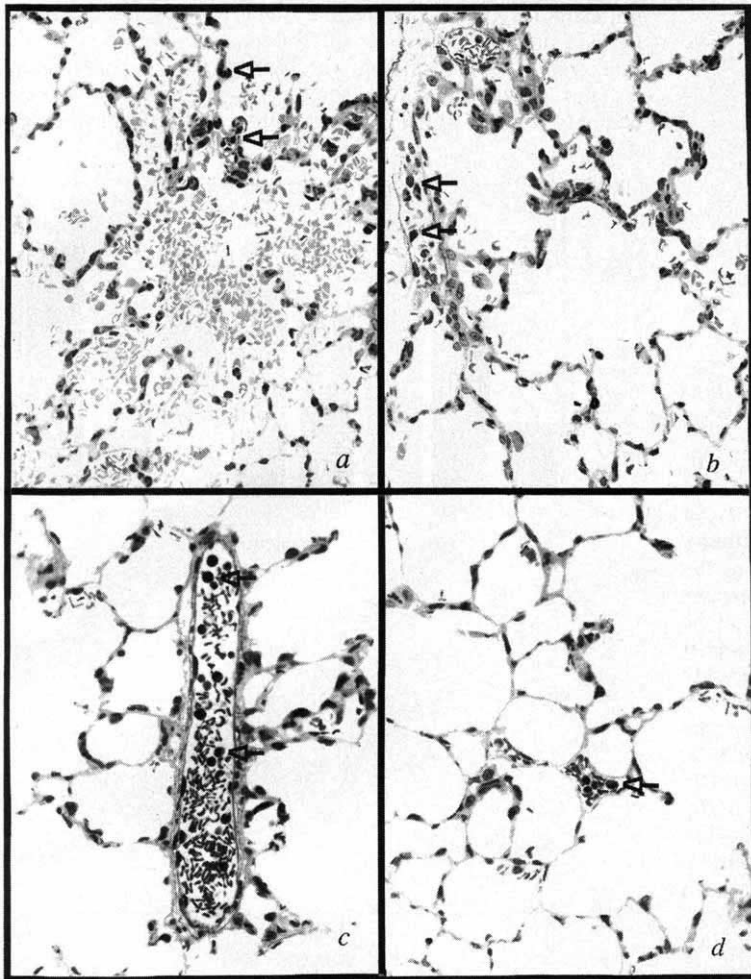


FIG. 4 Light micrographs of lungs from rats infused 30 min earlier with CVF and treated (5 min before infusion of CVF) with 200  $\mu$ g SLN-penta (a, b) or with 200  $\mu$ g SLX-penta (c, d), as described in Fig. 3. In a and b, there is extensive intra-alveolar haemorrhage; neutrophils (arrows) are close to the endothelial surfaces of interstitial capillaries (a) and venules (b). In animals treated with SLX-penta, there is little alveolar haemorrhage (c, d), and the intravascular neutrophils (arrows) are not close to the endothelium of venules (c) or interstitial capillaries (d). Preparation of morphological specimens is described elsewhere<sup>5</sup>. (Toluidine blue, all magnifications  $\times 120$ .)

Because there is evidence that SLX is a ligand for both E- and P-selectin<sup>8-11</sup>, this raises the question as to whether or not it can be concluded that the protective effects of SLX in the CVF model of lung injury are exclusively P-selectin related. In CVF-infused rats pretreated with either E- or P-selectin-IgG chimaera, protection against injury occurred only in the case of P-selectin-IgG chimaera (M.S.M., S. R. Watson, C. Fennie and P.A.W., manuscript in preparation). These data support the concept that in the CVF model of lung injury the protective effects of SLX are related to its interaction with P-selectin and not with E-selectin.

In humans, the counterpart to the CVF model of acute lung injury may be adult respiratory distress syndrome (ARDS) in which neutrophils are present in alveoli, accompanied by evidence of oxidative inactivation of protein sulphhydryls<sup>12</sup>. It is likely that ARDS is accompanied by complement activation, resulting in generation of C3a and C5a anaphylatoxins, leading to subsequent release of histamine, which upregulates endothelial P-selectin<sup>3</sup>. If the CVF model of lung injury is truly related to events in human ARDS, the SLX oligosaccharides may have therapeutic potential in the treatment of ARDS.  $\square$

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