

Protective Effects of Sialylated Oligosaccharides in Immune Complex-induced Acute Lung Injury

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Summary

Using sialyl Lewis^x (SLX) oligosaccharides derived from fucosyl transferase-expressing cells or generated synthetically, the ability of these compounds to protect against acute lung damage after deposition of immunoglobulin (Ig)G or IgA immune complexes has been determined. The synthetic compounds were tetra- and pentasaccharide derivatives of SLX as well as the nonfucosylated forms of SLX as controls. In the IgG immune complex model of lung injury, which is E-selectin dependent, SLX preparations provided dose-dependent protective effects, as assessed by changes in lung vascular permeability and hemorrhage. Protective effects were associated with diminished tissue accumulation of neutrophils in lungs (as assessed by myeloperoxidase). Morphological assessment revealed reduced physical contact of neutrophils with the pulmonary vascular endothelium and reduced tissue accumulation of neutrophils. In the model of IgA immune complex-induced lung injury, which does not involve participation of neutrophils and is independent of the requirement for E-selectin, SLX preparations were not protective. These data suggest that, in neutrophil-mediated and E-selectin-dependent lung injury, SLX preparations provide significant, protective effects against inflammatory vascular injury. The ability to achieve antiinflammatory outcomes in vivo with appropriate oligosaccharides suggests a new approach to the blocking of acute inflammatory responses.

The selectin family of leukocyte-promoting molecules consists of three structurally homologous glycoproteins containing, in the extracellular region, a calcium-dependent lectin binding domain, an epidermal growth factor (EGF) region, and a series of repeating complement-binding-like domains (1–7). Endothelial cells can be stimulated to synthesize E-selectin (endothelial leukocyte adhesion molecule type 1 [ELAM-1]), resulting in its induced expression on the cell membrane in 2–4 h (3). On the other hand, activation of endothelial cells or platelets by thrombin or histamine leads to rapid expression on the cell membrane of P-selectin (GMP-140, PADGEM), which is translocated from storage granules. Both E- and P-selectin react with the sialyl Lewis^x (SLX)¹ oligosaccharide and chemically related oligosaccharides (8–11). SLX expressed by neutrophils appears to be important in their adhesion to activated endothelial cells via E-selectin pathways (8). Engagement of selectin pathways is considered to be the first critical adhesion pathway that is associated with the rolling phenomenon of neutrophils along the endothelial surfaces

(12–14). Selectin engagement appears to be sequentially followed by participation of β_2 integrin molecules on neutrophils, together with their “counter-receptor” on the endothelial cells, intercellular adhesion molecules 1 and 2 (ICAM-1 and -2), after which firm attachment of leukocytes to the endothelial surface and transmigration of neutrophils to extravascular sites occur (15).

Using blocking mAbs, we have recently demonstrated that E-selectin is critical for the development of acute lung injury in rats after deposition of IgG immune complexes (16). In this model, lung vascular injury is critically dependent upon activation of neutrophils, their physical contact with and adherence to the endothelium, and generation of toxic oxygen products (17). In the current studies we have infused SLX oligosaccharides intravenously and have demonstrated significant protective effects against lung injury in the IgG immune complex-induced model of E-selectin-dependent lung injury. These protective effects were correlated with reduced adherence of neutrophils to the endothelium and diminished tissue accumulation of neutrophils. In contrast, in IgA immune complex-induced lung injury, which is independent of the requirements for E-selectin and neutrophils, SLX preparations

¹ Abbreviations used in this paper: DSA, *Datura stramonium* agglutinin; MPO, myeloperoxidase; SLX, sialyl Lewis^x.

were not protective. These findings suggest that SLX oligosaccharides may be a useful approach in blocking injury in the acute lung inflammation that is E-selectin dependent.

Materials and Methods

Reagents. Except where mentioned, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Adult male (300-gm) specific pathogen-free Long-Evans rats purchased from Charles River Labs (Portage, MI) were used in the studies.

Preparation of Glycopeptides. Cultured transfected Chinese hamster ovary (CHO) cell lines served as a source for glycopeptides. SLX-positive glycopeptides were prepared from an SLX-positive CHO cell line transfected with a human $\alpha(1,3/1,4)$ fucosyltransferase cDNA (CHO-FT cells) (11). Control, SLX-negative glycopeptides were prepared from an SLX-negative Chinese hamster ovary cell line transfected with a control vector without a cDNA insert (CHO-V cells). Conditions for growing these cell lines have been described elsewhere (11).

For preparation of [^3H]galactose-labeled glycopeptides from CHO-FT cells for oligosaccharide structural analysis, CHO-FT cells (plated at a density of 5×10^6 cells per 150-mm dish) were labeled for 24 h in α -MEM containing 10% FCS, 1% glucose, and 250 μCi [^3H]galactose (25.5 Ci/mmol; Amersham Corp., Arlington Heights, IL). After labeling, the cell monolayers were washed and the cells were harvested and processed exactly as noted above for the preparation of glycopeptides. The glycopeptides were fractionated by gel chromatography and were subjected to structural analysis by serial lectin affinity chromatography, exo- and endoglycosidase digestions, and methylation analyses. An aliquot of each expanded cell line was subjected to flow cytometry analysis at the time of harvest to ensure that the appropriate cell surface glycoconjugate phenotypes were present. The CHO-FT cells remained uniformly SLX positive, whereas the CHO-V cells were SLX negative, as expected. In addition, two confluent 150-mm dishes of each cell line were radiolabeled with [^{14}C]galactose (61 mCi/mmol; Amersham Corp.) to allow detection by scintillation counting of glycopeptides that were chromatographically isolated. Cells were radiolabeled by incubating the monolayers for 20 h in media containing [^{14}C]galactose (5.5×10^6 cpm).

Confluent CHO-FT or CHO-V cell monolayers were removed from the plates with PBS containing 3 mM EDTA (11). The radiolabeled cell monolayers were harvested in parallel with the nonlabeled monolayers and pooled with the nonlabeled cells. The cells were then pelleted and subjected to a series of solvent extractions before pronase digestion, as described previously (18–20). Briefly, the cell pellets were extracted three times with chloroform/methanol (2:1), once with methanol, and then twice with water. The pellet remaining after the last extraction was resuspended in 100 mM Tris, pH 7.4, 1 mM CaCl_2 . Pronase (pronase E, type XIV) was added to a final concentration of 25 mg/ml, and the mixture was digested for 16 h at 37°C. The mixture was then subjected to two additional periods (16 h) of digestion with pronase, at which time the mixture was supplemented with additional pronase (3.3 mg/ml of pronase added). After the last digestion, insoluble material (~ 1.5 -ml vol per 15-ml digestion) was removed by centrifugation, and the supernatant fluid was incubated at 100°C for 5 min. The supernatant fluid was then filtered through a 0.4- μm cellulose acetate filter, frozen, thawed, and filtered again. The solution was subsequently subjected to gel filtration chromatography through a Sephadex G-25 column (2.5 \times 20 cm) equilibrated in 5%

isopropanol in water, at a rate of 1 ml/min, with 100- μl fractions being collected. Each aliquot was subjected to scintillation counting. Fractions within the peak corresponding to the excluded volume (glycopeptides) were pooled, lyophilized, and were resuspended in 2.5 ml of water. The protein concentration of the pooled glycopeptides was determined using the anthrone method (21). The SLX-positive preparation was adjusted to a concentration of 1.39 mg/ml (reducing sugar concentration) with a protein concentration of 270 $\mu\text{g}/\text{ml}$. The control, SLX-negative material was resuspended at a concentration of 1.59 mg/ml with a protein concentration of 290 $\mu\text{g}/\text{ml}$. These labeled glycopeptides were analyzed after fractionated through Con A-Sepharose, followed by *Datura stramonium* agglutinin (DSA)-agarose chromatography (18). The glycoproteins that did not bind to these two columns represent N-glycans enriched with SLX structures. In addition, glycopeptides that were bound to DSA-agarose were also analyzed since they represent N-glycans with *N*-acetylglucosamine repeats.

To estimate the amount of terminal structures in the glycopeptides, the glycopeptide fractions were sequentially digested with diplococcal β -galactosidase and diplococcal β -*N*-acetylhexosaminidase (Exp. 1). Another portion of glycopeptides were digested with $\alpha(1,3/1,4)$ -specific fucosidase (19) followed by β -galactosidase and β -*N*-acetylglucosaminidase (Exp. 2). In separate experiments, the glycopeptides were first digested with Newcastle disease virus neuraminidase and asialo-glycopeptides were separately treated with exoglycosidases as described for intact glycopeptides (Exps. 3 and 4). The glycopeptides were subjected to Sephadex G-50 gel filtration before and after digestion, and the released galactose was determined in relation to the total radioactivity recovered after gel filtration.

The amount of galactose released in Exps. 1 and 2 corresponded to the amount of $\text{Gal}\beta(1,4)\text{GlcNAc}$ and $\text{Gal}\beta(1,4)(\text{Fuc}\alpha(1,3))\text{GlcNAc}$. The amount of galactose released in Exps. 3 and 4 corresponded to the amount of $\text{NeuNAc}\alpha(2,3)\text{Gal}\beta(1,4)\text{GlcNAc}$ and $\text{NeuNAc}\alpha(2,3)\text{Gal}\beta(1,4)(\text{Fuc}\alpha(1,3))\text{GlcNAc}$, respectively. This estimation is possible because the $\alpha(1,3/4)$ fucosidase was found to barely hydrolyze the fucosidic linkage in the SLX structure, but hydrolysis readily occurs with $\text{Gal}\beta(1,4)(\text{Fuc}\alpha(1,3))\text{GlcNAc}$ (18) (see Table 1).

74% of ^3H -labeled glycopeptides from CHO-FT cells failed to bind to Con A-Sepharose and DSA-agarose. From these glycopeptides, 3.9, 2.0, 16.3, and 29.1% of the total radioactivity was enzymatically released in Exps. 1, 2, 3, and 4, respectively. The results indicate these glycopeptides contain, 0.15, 0.08, 0.65, and 1.16 mol of $\text{Gal}\beta(1,4)\text{GlcNAc}$, $\text{Gal}\beta(1,4)(\text{Fuc}\alpha(1,3))\text{GlcNAc}$, $\text{NeuNAc}\alpha(2,3)\text{Gal}\beta(1,4)\text{GlcNAc}$, and $\text{NeuNAc}\alpha(2,3)\text{Gal}\beta(1,4)(\text{Fuc}\alpha(1,3))\text{GlcNAc}$ per molecule of the glycopeptides, respectively. The results also indicate these glycopeptides contain, on average, 2 mol of *N*-acetylglucosamine repeats, since only 50% of the total radioactivity was removed by one cycle of the sequential enzyme digestion. The results obtained on DSA-bound glycopeptides (11.4% of the total radioactivity) were similarly calculated.

Animal Models of IgG and IgA Immune Complex-induced Alveolitis. Rabbit polyclonal IgG rich in antibody to BSA (anti-BSA) was used in the first model. IgG was purchased from Organon Technika (Westchester, PA). Intraperitoneal ketamine was administered for anesthesia. 2.5 mg anti-BSA in a volume of 300 μl was intratracheally instilled via a small intratracheal catheter during inspiration after surgical exposure of the trachea. Intravenous injection of 10 mg BSA together with trace amounts of ^{125}I -BSA and ^{51}Cr -RBC, as described in detail elsewhere (16), was used. Rats were killed 4 h later and lung injury was quantitated by permeability and hemorrhage measurements. For induction of IgA immune complex-induced lung injury, murine myeloma IgA pro-

tein, which is reactive with dinitrophenol (DNP), was isolated as previously described (22). 1.2 mg IgA anti-DNP was instilled intratracheally and 3.3 mg DNP-BSA injected intravenously together with ⁵¹Cr-RBC and trace amounts of ¹²⁵I-BSA, as described above. As with the IgG immune complex model of lung injury, injury was measured at 4 h and assessed by increased vascular permeability and hemorrhage. As described above, protective interventions involved the intravenous injection of oligosaccharides at 2.5, 3.0, and 3.5 h after initiation of IgA immune complex deposition in lung.

When used in vivo, 400 μ l of the CHO-FT preparation (and of the composition described in Table 1) was injected intravenously in three equally divided doses at 2, 2.5, and 3 h after deposition of IgG immune complexes. This schedule for injections was selected since it coincides with the time during which rapid expression of E-selectin occurs in the pulmonary vasculature (16). This dose contained 556 μ g carbohydrate and 108 μ g protein. Reference-positive controls received PBS or 400 μ l CHO-V preparation in a similar injection schedule. In the latter, the injected preparation contained 636 μ g carbohydrate and 116 μ g protein. The purified SLX preparation with reducing sugar (SLX-OH) as well as the tetrasaccharide and pentasaccharide carbonyl derivatives of SLX were prepared biosynthetically as described in a recent paper (23). When used, indicated doses of SLX(OH) or tetrasaccharide or oligosaccharide carbonyl derivative were also injected intravenously at 2.5, 3.0, and 3.5 h after initiation of the immune complex-induced lung inflammatory models of injury.

Synthetic Oligosaccharides. Synthetic derivatives of SLX and related nonfucosylated analogues were prepared by combined chemical and enzymatic synthesis (23, 24). Compounds used in this report included reducing sugars, NeuAc α 2,3Gal β 1,4GlcNeuAc (SLN-OH) and NeuAc α 2,3Gal β 1,4(Fuc α 1,3)GlcNAc (SLX-OH); and glycosides NeuAc2, 3Gal β 1,4(Fuc α 1,3)GlcNAc- β -0(CH₂)COOCH₃ (SLX-tetra) and NeuAc α 2,3Gal β 1,4 (Fuc α 1,3)GlcNAc β 1,3Gal β -0-(CH₂)₅COOCH₃ (SLX-penta).

Tissue Myeloperoxidase (MPO) Activity. To assess the use of tissue MPO activity as a measure of neutrophil influx, known numbers of glycogen-elicited rat peritoneal neutrophils were instilled into the airways of normal rat lungs, the tissue was homogenized and extracted, and standard curves were produced as previously described (16). Lung samples obtained 4 h after deposition of immune complexes were homogenized with a polytron homogenizer (Techmar Co., Cincinnati, OH) using 6 ml of homogenization buffer (50 mM phosphate, pH 6.0) and subjected to centrifugation (3,000 g; 30 min at 4°C). MPO activity in supernatant fluids was assayed by measuring the change in absorbance at 460 nm resulting from oxidation of *o*-dianisidine in the presence of H₂O₂.

Morphologic Evaluation of Lungs and Skin. Lungs were fixed in

glutaraldehyde and embedded in epon and processed for light microscopy in the conventional manner.

Statistical Analysis. Data were analyzed using one- or two-way analysis of variance (ANOVA) for the individual experiments. If significant differences were detected among group means, individual group means were then compared with positive controls so as to discern statistical differences within data sets, using the Scheffe *t* test and the Fisher protected least significant difference (PLSD) comparison tests. All values were expressed as mean \pm SEM, unless otherwise indicated. Statistically, significant differences were defined as *p* < 0.05. For calculation of percent protection, negative control values were subtracted from both positive control values and from values of treated protective control groups.

Results

Analysis of CHO Cell-expressed Oligosaccharides. Based on the average molecular weight of glycopeptides (3,750 for Con A-DSA unbound and 2,280 for DSA bound) and the amount used in in vivo experiments (186 μ g/injection), the amount of each terminal structure can be calculated as shown in Table 1. The CHO-FT (fucosyl transferase-transfected cells) cell glycopeptides used for in vivo experiments contained the composition as described in Table 1. The predominant species was SLX, with lesser amounts of sialyl LacNAc, LacNAc, and Lewis^x, in descending order. For each of the three intravenously injected doses (at 2, 2.5, and 3 h), the amounts (nmol) per injection of oligosaccharide are shown in Table 1. Assuming distribution of the oligosaccharides into a total blood volume of 15 ml and excluding equilibration with the extravascular compartment, a single-dose oligosaccharide would yield a maximal blood concentration of 9 μ M SLX. The other oligosaccharides would be at a much lower concentration.

Ability of Glycopeptide Fractions from Fucosyl Transferase-transfected and SLX-expressing Cells to Attenuate IgG Immune Complex-induced Lung Injury. IgG immune complex-induced lung injury was quantitated at 4 h by vascular permeability (Fig. 1 A), hemorrhage (B), and by MPO content (C). Recent studies have indicated that this model of lung injury is E-selectin dependent (16). When the positive control group receiving intravenous injections of PBS (at 2.5, 3.0, and 3.5 h) was compared with the group similarly infused with 636 μ g glycopeptide carbohydrate from CHO-V (vector-transfected) cells, no reduction in any of the three parameters was noted.

Table 1. Oligosaccharide Structural Analysis of Concentrates Used for In Vivo Treatment

Structure	Trivial name	Mass concentration	Amount injected*
		(hexose in glycopeptide)	
		nmol/ μ g	nmol
NeuNAc α 2,3Gal β 1,3(Fuc α 1,3)GlcNAc	SLX	0.715	132.5
Gal β 1,4(Fuc α 1,3)GlcNAc	Lewis ^x	0.072	13.3
NeuNAc α 2,3Gal β 1,4GlcNAc	sialyl LacNAc	0.402	74.5
Gal β 1,4GlcNAc	LacNAc	0.122	22.6

* Approximately 185.3 μ g hexose was administered per injection, and a total of three doses were given intravenously at 2.5, 3.0 and 3.5 h.

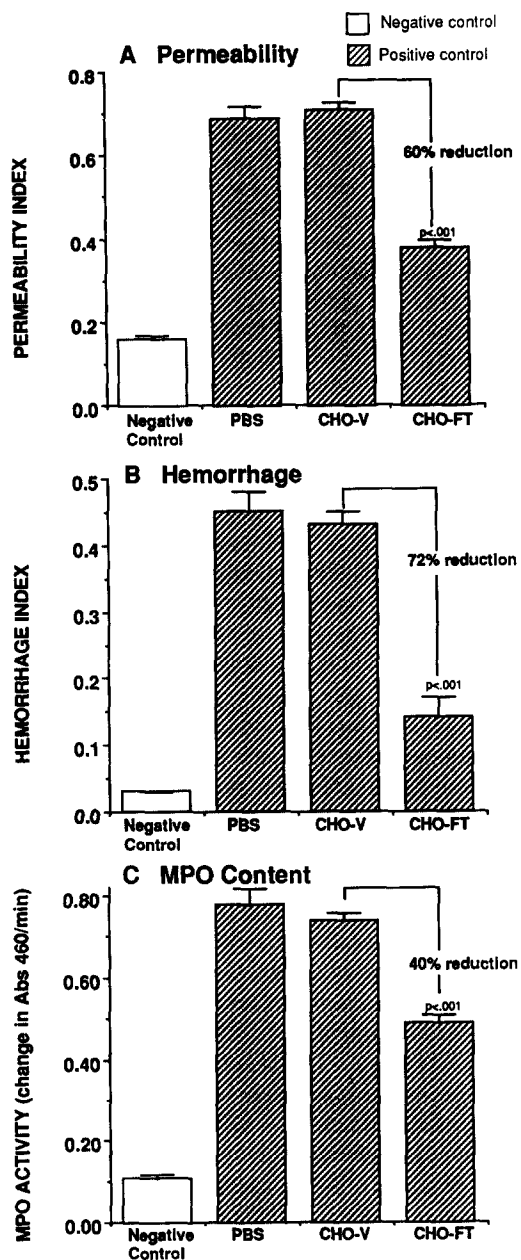


Figure 1. IgG immune complex-induced acute lung injury in rats 4 h after intrapulmonary deposition of IgG immune complexes. Injury was measured by the increase in vascular permeability (A) or hemorrhage (B). Lung content of MPO was also determined (C). The negative permeability, hemorrhage, and MPO values in the negative controls (animals in which the intravenous injection of BSA was omitted) were: 0.16 ± 0.01 , 0.03 ± 0.001 , and 0.11 ± 0.01 , respectively. Values for positive reference control groups (animals receiving PBS instead of an oligosaccharide infusion) for permeability, hemorrhage, and MPO were: 0.68 ± 0.03 , 0.45 ± 0.03 , and 0.76 ± 0.04 , respectively. CHO-V was the carbohydrate-rich product from CHO cells transfected with vector alone (CHO-V cells) while CHO-FT was the carbohydrate-rich product from CHO cells transfected with the cDNA for $\alpha(1,3/1,4)$ fucosyltransferase (CHO-FT cells). Details are provided in the text. For each vertical bar, $n = 6$.

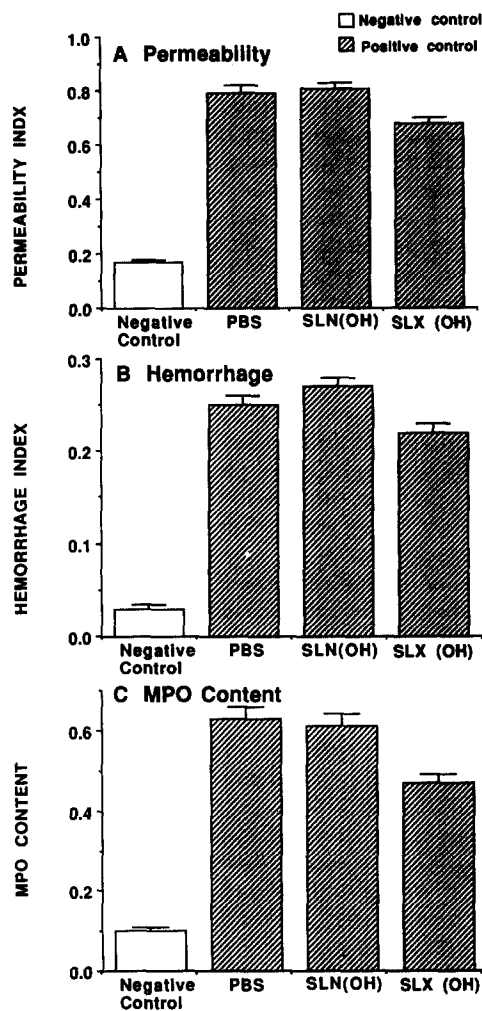


Figure 2. Protective effects of natural preparations (injected in equally divided doses intravenously at 2.5, 3.0, and 3.5 h) of SLX reducing sugar (SLX-OH) and the nonfucosylated oligosaccharide (SLN-OH) in IgG immune complex-induced lung injury. Composition of material infused is described in Table 1 and in the first section of Results. Positive control values (PBS-infused rats) for permeability (A), hemorrhage (B), and MPO (C) parameters were: 0.79 ± 0.03 , 0.25 ± 0.01 , and 0.63 ± 0.03 , respectively. Details of treatment are provided in the text. For each vertical bar, $n = 6$.

In contrast, in rats treated with 556 μg of glycopeptide carbohydrate prepared from the cell line expressing SLX (CHO-FT), the permeability index was reduced by 60% ($p < 0.001$), hemorrhage by 72% ($p < 0.001$), and MPO content in lung fell by 40% ($p < 0.001$), indicating the protective effects of this reagent and a correlative reduction in lung MPO content. Based on the reduction of lung injury observed with natural carbohydrate containing SLX, synthetic preparations of SLX and related oligosaccharides were used in subsequent experiments.

Reduction in IgG Immune Complex-induced Lung Injury by Synthetically Generated SLX Oligosaccharides. Synthetic SLX reducing sugar (SLX-OH) or the nonfucosylated control (SLN-OH) was administered intravenously at 2.5, 3.0, and 3.5 h after intrapulmonary deposition of immune complexes.

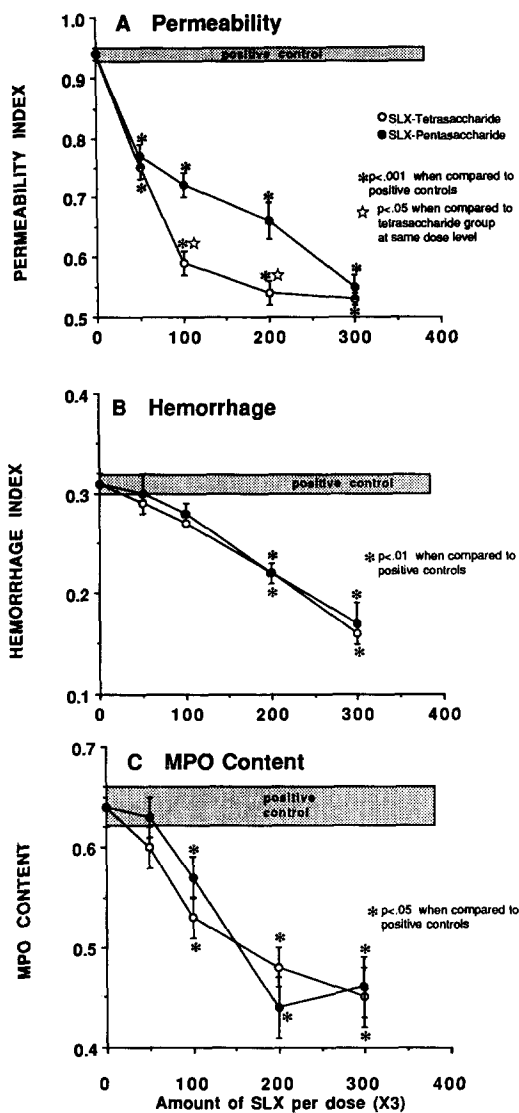


Figure 3. Ability of tetrasaccharide and pentasaccharide (glycoside) derivatives of SLX to reduce lung vascular injury after intrapulmonary deposition of IgG immune complexes. Oligosaccharides were infused intravenously in the indicated doses at 2.5, 3.0, and 3.5 h after intrapulmonary deposition of IgG immune complexes. Positive control values (PBS-treated rats) for permeability (A), hemorrhage (B), and MPO (C) parameters were 0.94 ± 0.01 , 0.31 ± 0.01 , and 0.64 ± 0.02 , respectively, and are shown by the stippled horizontal bar. Details of treatment are provided for in the text. For each data point, $n = 6$.

200 μg was injected at each of the three time points. Measurements of lung vascular injury were made at 4 h together with lung content of MPO. All comparisons were made to animals that had been treated intravenously with PBS instead of an oligosaccharide. These reference-positive control values (PBS), expressed as mean \pm SEM, are shown in the bars in Fig. 2, A–C. When compared with results obtained in positive control animals that had been treated with PBS, treatment of rats with SLN-OH showed no alteration in vascular permeability value (Fig. 2, A), no decrease in hemorrhage (B), and no decrement in lung content of MPO (C). In contrast, treatment with similar amounts of SLX-OH caused

modest reductions in permeability (20%, $p = 0.004$), hemorrhage (21%, p is not significant), and in MPO content (27%, $p = 0.005$).

Also examined were tetra- and pentasaccharide glycosides of SLX using a range of doses (50–500 μg) with intravenous injections of the indicated amounts at 2.5, 3.0, and 3.5 h. Reference-positive controls received 200 μg SLN-OH intravenously as the interventional agent; the values for these groups are shown by the horizontal bars (mean \pm S.E.M.) indicated in each of the panels (Fig. 3). At all concentrations of SLX preparations used, there were significant reductions in lung injury as reflected by diminished vascular permeability. Over the dose range of 50, 100, 200, and 300 μg (injected in equal doses at each of the three time intervals indicated), treatment with the SLX-tetrasaccharide preparation reduced injury (as measured by permeability change) by 24% ($p < 0.001$), 45% ($p < 0.001$), 51% ($p < 0.001$), and 53% ($p < 0.001$), respectively (Fig. 3 A). In the case of the SLX-pentasaccharide preparation, the reductions in permeability were 22% ($p < 0.001$), 28% ($p < 0.001$), 36% ($p < 0.001$), and 50% ($p < 0.001$), respectively. At the 100- and 200- μg doses, the SLX-tetrasaccharide was significantly more protective than the SLX-pentasaccharide ($p = 0.02$ and 0.045 , respectively). When the effects of these oligosaccharides on hemorrhage were evaluated, only treatment with the two highest doses of tetrasaccharide and pentasaccharide SLX preparations produced statistically significant protection. For the SLX-tetrasaccharide at the doses of 200 and 300 μg , injury was reduced 33% ($p = 0.004$) and 56% ($p < 0.001$), respectively; for the SLX-pentasaccharide, at the same doses, injury was 33% ($p = 0.011$) and 52% ($p = 0.001$), respectively (Fig. 3 B). With respect to the effects of the oligosaccharides on lung MPO content after deposition of IgG immune complexes, treatment with 50 μg of either the SLX-tetra- or pentasaccharides (at 2.5, 3.0, and 3.5 h) did not significantly reduce MPO content, but at the 100-, 200-, and 300- μg doses of the SLX-tetrasaccharide, reductions in MPO content were 21% ($p = 0.01$), 30% ($p = 0.004$), and 36% ($p = 0.003$), respectively, while treatment with the SLX-pentasaccharide preparation reduced MPO content by 11% ($p = 0.048$), 38% ($p = 0.002$), and 34% ($p = 0.004$), respectively. At any treatment when the SLX-penta- and tetrasaccharides were compared with each other there were no significant differences in MPO content. Thus, the protective effects of the tetra- and pentasaccharide preparations in the IgG immune complex model of lung injury are dose dependent and roughly correlate with reductions in lung content of MPO.

Morphological Correlates of Oligosaccharide-induced Lung Protection. The features of IgG1 immune complex-induced acute lung injury 4 h after immune complex deposition in lungs are shown in Fig. 4 A, in which tissue was derived from a positive control treated intravenously with 200 μg SLN-OH at 2.5, 3.0, and 3.5 h. There was extensive intraalveolar hemorrhage, and the venules and capillaries contained numerous neutrophils, many of which were in direct contact with the endothelial surfaces (arrows). In animals treated with 200 μg SLX-tetrasaccharide at the same intervals of time, there was marked suppression of hemorrhage and, of the rel-

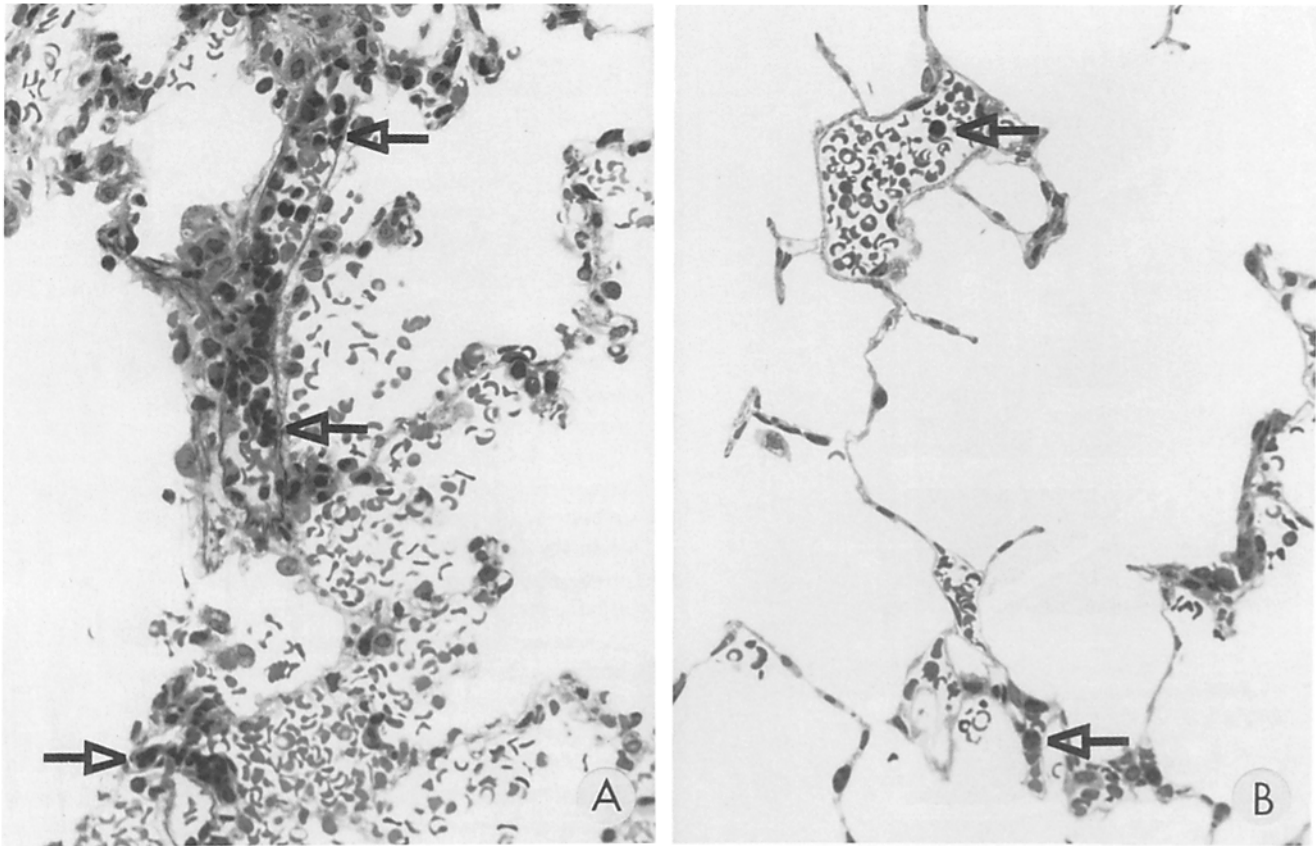


Figure 4. IgG immune complex-induced alveolitis at 4 h. In *A*, from an animal treated with 200 μ g SLN-OH at 2.5, 3.0, and 3.5 h, there was extensive intraalveolar hemorrhage. Within the venules and capillaries many neutrophils were in direct contact with the vascular endothelium (arrows). In animals treated with 200 μ g SLX-tetrasaccharide using the same protocol, little alveolar hemorrhage developed and, of the intravascular neutrophils present, few were in contact with the endothelium (arrows). (Toluidine blue-stained sections, $\times 115$.)

atively few neutrophils that were present within the vascular compartment, they were within the vascular lumens and rarely in direct contact with the endothelial surfaces (Fig. 4 *B*, arrows).

Inability of SLX-OH to Protect against IgA Immune Complex-induced Lung Injury. The reducing sugar form of SLX (SLX-OH), which was protective in IgG immune complex-induced injury (Fig. 2), was also used in the IgA immune complex model of lung injury. In this model neutrophil recruitment is not involved in the injury-inducing events in lung (22) and E-selectin is not required for development of lung damage (25). Injury in this model appears due to generation of toxic oxygen and L-arginine-derived products from lung macrophages (25, 26). As in the case of the IgG immune complex model of injury (Fig. 1). PBS, SLN, or SLX-OH were infused intravenously at 2.5, 3.0, and 3.5 h after deposition of IgA immune complexes. The composition of the infused material is described in Table 1 and above. Permeability and hemorrhage parameters increased by two- to threefold when positive controls (treated with PBS) were compared with negative controls (animals receiving an airway instillation of IgA antibody but no DNP-BSA intravenously [Fig. 5]). When positive control animals were treated intravenously with either SLN or SLX-OH at 2.5, 3.0, and 3.5 h, there was no

reduction in the intensity of injury, measured either by permeability change (Fig. 5 *A*) or hemorrhage (*B*). As indicated above, this is in contrast to the protective effects of these oligosaccharides in lung injury induced by intrapulmonary deposition of IgG immune complexes (Fig. 2). MPO data are not shown since, in this model, few neutrophils accumulate in lung and neutrophil depletion is not protective (26).

Discussion

There is increasing evidence for the role of selectins in the inflammatory response. The *in vitro* use of perfusion chambers that permit visual observations of neutrophil-adhesive interactions to selectin-bearing surfaces under conditions of shear stress has led to the hypothesis that interactions of P- or E-selectins with "counter-receptors" on neutrophils can result in the initial rolling of leukocytes along a selectin-bearing surface (12, 13). This rolling is thought to be due to intermittent adhesive interactions between neutrophils and endothelial cells, resembling the earliest events *in vivo* in the acute inflammatory reaction during which time neutrophils convert from a rapid luminal flow to a "margination," which is manifested as a rolling along the endothelium at a rate that

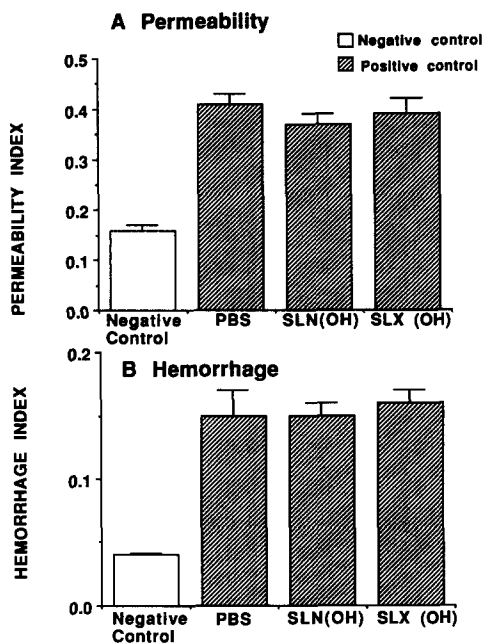


Figure 5. Protective effects of natural preparations (200 μ g injected intravenously at 2.5, 3.0, and 3.5 h) of SLX-OH and the nonfucosylated SLN-OH in IgA immune complex-induced acute lung injury. Composition of material infused is described in Table 1 and in the first section of Results. Negative and positive (PBS-treated) control values for permeability were 0.16 ± 0.01 and 0.37 ± 0.02 , respectively. Comparison values for hemorrhage were 0.04 ± 0.001 and 0.15 ± 0.01 , respectively. For each vertical bar, $n = 4$.

is much slower than that of cells being carried along by the luminal flow of blood. After the selectin-dependent rolling phenomenon, it appears that the next sequence of adhesion-promoting interactions involves β_2 integrins of the neutrophil and the counter-receptors on the endothelial cell, ICAM-1 or -2 (15). Integrin-mediated adhesion appears to result in a tight adhesive interaction between neutrophils and the endothelium, producing a cessation of rolling, a flattening and spreading on the endothelium of the neutrophil, and the movement beyond the endothelial barrier (through intercellular junctions) of the neutrophil (13).

The *in vivo* evidence documenting the requisite role of selectins in the inflammatory response is limited. There is experimental evidence suggesting that in animals E-selectin may be a homing molecule for a subclass of T cells (27). Expression of endothelial E-selectin (using immunohistochemical analysis) has been demonstrated in human skin obtained from patients with contact hypersensitivity reactions (28, 29), in the vasculature of synovial tissues in patients with rheumatoid arthritis (30) and in multiple vascular beds of baboons infused with LPS (31, 32), providing indirect evidence for the possible involvement of E-selectin in these inflammatory conditions. In IgG immune complex-induced dermal and lung vasculitis, vascular expression of rat E-selectin (ELAM-1) has been shown immunohistochemically, and protective effects (which have been associated with greatly reduced neutrophil influx) have been obtained by blocking of E-selectin with F(ab')₂ preparations of anti-E-selectin (16). Blocking of

L-selectin by the use of antibody (33) or by the use of a L-selectin-Ig chimeric molecule (34) has reduced the influx of neutrophils into the peritoneal cavity of mice. Sialidase treatment of animals or the use of carbohydrates that prevent adhesive interactions between T cells and high endothelial cells of lymph nodes also interferes with the recirculation (from blood to tissues and ultimately back to the blood compartment) of T cells (35), suggesting that L-selectin may also be an important participant in inflammatory responses featuring the participation of neutrophils, T cells, or monocytes.

Compelling evidence for the importance of E-selectin and P-selectin interaction with their carbohydrate ligand, SLX, on neutrophils comes from the recent description of a new leukocyte adhesion deficiency stemming from the absence of SLX on patients' neutrophils (36). Although not as severe as the classic leukocyte adhesion deficiency, which features a defect in the neutrophil CD18 integrins, the pronounced deficiency exhibited by SLX-deficient individuals results in recurrent severe infections and high neutrophil counts in blood. Thus, it is reasonable to assume that disruption of the E- and P-selectin interactions with SLX-containing ligands on the neutrophil will also prevent neutrophil migration into tissues in inflammatory conditions and prevent resultant neutrophil-mediated damage.

In this study, we have addressed the potential for soluble forms of the E-selectin ligand, SLX, to inhibit an E-selectin-dependent model of inflammation, the acute lung injury after the acute inflammatory vascular injury after deposition of IgG immune complexes. In this model of injury, engagement and activation of neutrophils is crucial. Neutrophil migration into the alveolar compartment containing IgG immune complex deposits results in protease release and generation of nitrogen- and oxygen-centered free radicals. Pulmonary macrophages also contribute to the outcome of injury of vascular endothelial and alveolar epithelial cells by their production of oxidants and their release of cytokines that appear to upregulate endothelial adhesion molecules (17, 37, 38). On the basis of the data in the current report, the infusion of natural N-linked oligosaccharides containing SLX or synthetic derivatives of SLX causes significant reduction of lung injury and a corresponding reduction in neutrophil accumulation in lung tissue. The incomplete protection by SLX compounds against this type of injury could be due to a variety of factors, including SLX oligosaccharide blood levels that are insufficient to compete totally with the *in vivo* presentation of SLX on the neutrophil. Other explanations could be the rapid engagement of the β_2 integrin/ICAM-1, -2 pathway, or injury due to nonneutrophil-dependent mechanisms. With respect to the last possibility, the IgA immune complex model of acute lung injury is informative. In this model of lung injury, it is known that little neutrophil recruitment occurs; pulmonary macrophages appear to be the key effector cells in events leading to pulmonary injury. In this model, little vascular expression of E-selectin is found and blocking antibody to E-selectin has no protective effects (in striking contrast to findings in the IgG immune complex model) (25). Similarly, as presented in Fig. 5, SLX oligosaccharides also have no protective effects in this model.

The ability of SLX oligosaccharides to protect against neutrophil-mediated acute lung injury, as documented in this report, suggests that the development of SLX-bearing conjugates may be effective antiinflammatory approaches. Critical needs at present are access to more effective glycocon-

jugates with prolonged half-lives in the circulation, a clearer definition of the interactions between oligosaccharides and selectins, and more information regarding inflammatory conditions in which selectins are playing an important role in the development of the inflammatory response.

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