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Glycosaminoglycan Composition of Germfree Dog Lungs[1] (41587)

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Abstract. Connective tissue of the lung is fundamentally important to pulmonary function. Exposure to foreign materials such as environmental toxins and microorganisms alters the connective tissue composition of pulmonary structures. In an effort to obtain information on the nature of complex carbohydrates in lungs unexposed to foreign materials, the composition of glycosaminoglycans from germfree dogs was studied. The total glycosaminoglycan concentration in germfree dog lungs was 47% greater than that in conventional dog lungs. Variations in individual glycosaminoglycan concentrations between germfree dog lungs and conventional dog lungs were noted. Heparan sulfate and heparin concentrations were lower in germfree dog lungs than in conventional dog lungs. Heparan sulfate fractions from germfree dog lungs had lower total sulfate content than heparan sulfate fractions from conventional dog lungs. The differences in glycosaminoglycan composition between germfree dog lungs and conventional dog lungs are likely due to exposure of conventional animals to a "normal" microbial flora and other environmental factors.

Very little is known of the complex carbohydrates of connective tissue in germfree animals. Earlier studies of carbohydrate–protein macromolecules in germfree animals focused mostly on intestinal mucosa. Lindstedt *et al.* (1) noted that hexosamine content of mucus in the cecum of germfree rats was five times greater than in the cecal mucosa of conventional rats. Most of the hexosamine was present in a high molecular-weight glycoprotein, suggesting an endogenous origin of the protein. In addition, Hoskins (2) observed that in the intestinal contents of germfree rats certain types of mucinases, normally present in conventional rats, were absent. It has been suggested by Loesche (3) that in the absence of microbes in these animals endogenous proteins are not degraded in the gut in a manner similar to that in conventional animals. Heneghan *et al.* (4) observed that intestinal mucus and mucus-producing goblet cells were significantly greater in germfree animals compared to conventional controls.

Carbohydrate–protein macromolecules play an important role in the function of respiratory system not only as structural support but as components of mucociliary system as well, which provides a barrier for foreign substances like microorganisms and inspired chemicals. Exposure to foreign materials that often occurs in conventional animals alters the composition of mucociliary system. A study of the composition of lungs from germfree animals provides information on the nature of carbohydrate–protein macromolecules in lungs unexposed to microorganisms. In the present study glycosaminoglycans (GAG) were isolated from germfree dogs, their composition was studied and compared with the composition of GAG from lungs of conventional control dogs.

Materials and Methods. *Animals—germfree.* Four germfree beagle dogs, three females and one male, aged 18 months and weighing between 20 and 30 lb were utilized. Germfree puppies were delivered by Caesarean section into germfree flexible film isolators, fed sterilized bitch's milk replacement and dog food, and maintained throughout their lives in isolation under germfree conditions, as previously described (5). Routine microbiological tests immediately prior to euthanasia confirmed their germfree status. Germfree-dog

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rearing and all subsequent experimental protocols followed guidelines of the American Physiological Society and were approved by the Louisiana State University Medical Center Animal Care Committee, while the animal care facilities were inspected and approved by the American Association for Accreditation of Laboratory Animal Care.

Four Mongrel dogs, three females and one male, weighing 20 to 30 lb were used as conventional controls.

Immediately following necropsy, lungs were obtained and dissected free of extraneous tissue. The lungs were finely minced quickly rinsed three times with cold 0.15 *M* NaCl, and dry-defatted over acetone (five changes) at 4°C over a period of 4 days. The dry-defatted tissue was stored at 4°C in a desiccator under vacuum.

Isolation of GAG. GAG were isolated from dry-defatted lung tissues by procedures previously described (6, 7). Briefly, the tissue was digested with papain at 65°C for 48 hr. An equal volume of 12% trichloroacetic acid (TCA) was added and the protein material precipitated; the latter was separated from GAG solution by centrifugation at 14,000*g* for 30 min. The supernatant was exhaustively dialyzed against distilled water to remove TCA and then lyophilized. This material was further hydrolyzed by pronase in 0.1 *M* phosphate buffer, pH 7.6, for 48 hr and GAG were isolated as previously described (7).

Fractionation of GAG. Fractionation of GAG was achieved on a Dowex-1 Cl^- column as previously described (8). The GAG from the column was eluted with an NaCl–$MgCl_2$ gradient (0.5 *M*–3.0 *M*). The fractions were analyzed by orcinol–H_2SO_4 reaction in a Technicon sugar analyzer. Fractions corresponding to peaks on the recorder were collected using a stream splitter in the manifold. These were pooled, dialyzed against distilled water, and analyzed. Relative concentrations of hyaluronic acid, heparan sulfate, and chondroitin sulfates plus heparin were calculated from areas under the peaks in the chromatograms. Isomeric chondroitin sulfates in mixtures were estimated by the procedure of Saito *et al.* (9) and heparin from glucosamine content in the fraction. The automated chromatography procedure of analysis of GAG was found reproducible and the results were comparable to those obtained by manual chromatography followed by analysis of individual fractions for characterization (7, 8). Replicate analyses showed a mean deviation of 2% from the average for hyaluronic acid, 4% for heparan sulfate, 7% for chondroitin 6-sulfate, 10% chondroitin 4-sulfate, 5% dermatan sulfate, and 7% heparin.

Gel filtration. Gel filtration of GAG fractions was performed on Sepharose CL-6B column as previously described (10). GAG from the column was eluted with 0.5 *M* sodium acetate, pH 5.8. The fractions were analyzed by orcinol–H_2SO_4 reaction in a Technicon sugar analyzer.

Electrophoresis. Electrophoresis of GAG was performed on cellulose acetate strips in pyridine/formate buffer, pH 3.6, as previously described (11). GAG were localized by Alcian blue stain.

Analyses. Hexuronic acid was determined by the Dische method (12) and hexosamine by the method of Boas (13) after the sample had been hydrolyzed by 4 *N* HCl for 14 hr at 100°C. Differential determinations of glucuronic acid and iduronic acid, glucosamine and galactosamine, and acetyl groups were done by gas–liquid chromatography procedures (14–16). Total sulfate was estimated by the rhodizonate procedure of Terho and Hartiala (17) and N-sulfate by nitrous acid degradation (18). Protein was determined by the procedure of Lowry *et al.* (19).

Results. The total uronic acid concentration and relative distribution of individual GAG in lungs from germfree and conventional controls are reported in Table I. GAG isolated by papain digestion of lung tissue contained considerable amount of protein material and therefore, GAG preparations were subsequently hydrolyzed by pronase. Pronase digestion resulted in GAG devoid of detectable protein by the Lowry *et al.* method (19). The mean total uronic acid concentration in lungs from germfree dogs was 47% greater than that in conventional dog lungs. Except one, all the germfree dog lungs had higher concentrations of uronate than the highest value in conventional control samples.

Figure 1 illustrates Dowex-1 Cl^- column chromatographic profiles of GAG from the lungs of germfree and conventional dogs. In

TABLE I. GAG COMPOSITION OF GERMFREE AND CONVENTIONAL DOG LUNGS

Group	Animal No.	μg UA[a]/mg tissue[b]	Percentage composition of total GAG					
			HA	HS	C-4-S	C-6-S	DS	Hep[c]
Germfree	1	2.6	8.5	35.9	12.2	31.8	7.5	4.1
	2	3.0	10.8	38.2	10.1	29.0	8.2	3.7
	3	1.9	6.4	39.1	13.4	30.9	7.7	2.5
	4	2.7	7.9	36.4	12.8	32.8	7.3	2.8
	Mean	2.5	8.4	37.4	12.1	31.1	7.7	3.3
	SE	0.25	0.90	0.75	0.70	0.80	0.20	0.40
Conventional	5	1.9	4.2	41.5	8.3	29.8	9.6	6.6
	6	1.4	3.5	43.2	9.6	29.1	7.2	7.4
	7	1.8	3.7	38.6	11.3	30.9	8.4	7.1
	8	2.0	5.1	42.7	8.2	27.5	10.8	5.7
	Mean	1.7	4.1	41.5	9.4	29.3	9.0	6.7
	SE	0.15	0.35	1.0	0.70	0.70	0.75	0.35
Significance[d] ($P<$)		0.05	0.005	0.025	0.05	NS	NS	0.001

[a] Abbreviations: UA—uronic acid; HA—hyaluronic acid; HS—heparan sulfate; C-4-S—chondroitin 4-sulfate; C-6-S—chondroitin 6-sulfate; DS—dermatan suflate; Hep—heparin; SE—standard error of the mean; NS—statistically not significant.

[b] Acetone dry-defatted tissue.

[c] May contain small amounts of keratan sulfate.

[d] Statistical significance—the difference between the two groups.

all samples GAG resolved into three peaks and the third peak was heterogenous. Cellulose acetate electrophoresis indicated that peak 1 corresponds to hyaluronic acid, peak 2 to heparan sulfate, and peak 3 to a mixture of chondroitin sulfates and heparin. Concentrations of individual GAG in each sample calculated from areas of the peaks and estimated by enzymatic and chemical analyses are reported in Table I. The major GAG in all samples were heparan sulfate (about 40%) and chondroitin 6-sulfate (about 30%). Chondroitin 4-sulfate constituted about 10% of the GAG. Hyaluronic acid and heparin were present in lesser amounts (3–10%).

Mean concentrations of hyaluronic acid and chondroitin 4-sulfate were significantly greater in germfree animals than in conventional controls. Hyaluronic acid in germfree dogs was twice as much as in controls. But heparan sulfate and heparin concentrations were significantly lower in germfree animals. Although the mean value of dermatan sulfate in germfree dogs was 16% less than in controls, the difference was not statistically significant.

The Dowex-1 Cl^- column chromatographic profiles of GAG from individual animals showed a difference in the relative position of heparan sulfate (peak 2). Fractions corresponding to this peak were extensively analyzed and the results are reported in Table II. Germfree dog lung heparan sulfate had lower total sulfate and N-sulfate, but the difference was statistically significant only in total sulfate content. There were no differences in the molar proportions of uronic acid and N-acetyl content to hexosamine. The ratio of iduronic acid to glucuronic acid was greater in conventional dogs than in germfree dogs.

No differences were noted in gel-filtration profiles of Dowex-1 Cl^- column fractions of GAG from germfree dog lungs and conventional controls. Hyaluronic acid from both samples eluted in the void volume of the column. Heparan sulfate from both germfree and controls eluted from the column with a K_{av} value of 0.49. Isomeric chondroitin sulfates and heparin gave broad heterogenous peaks with the major peak in the germfree animal sample eluting from the column with a K_{av} value of 0.56.

Discussion. The results indicate that germfree dogs have greater concentrations of GAG in their lungs than conventional dogs. Whether this higher concentration is due to increased

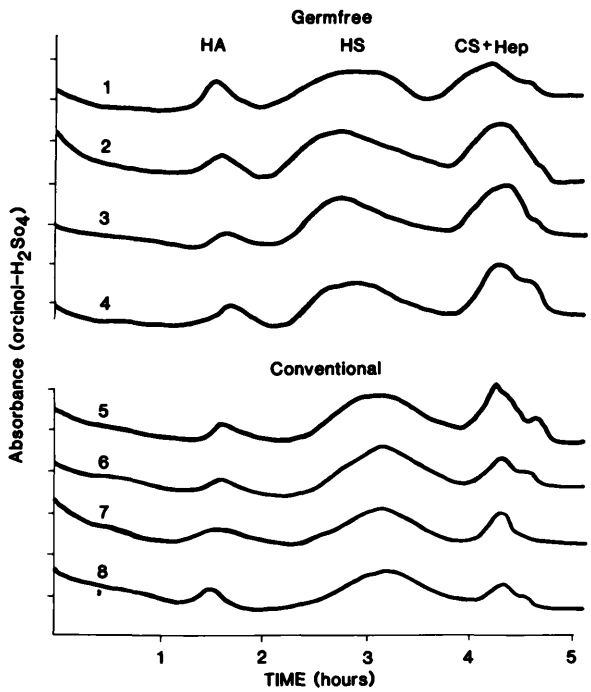

FIG. 1. A composite illustration of Dowex-1 Cl^- column-chromatography profiles of glycosaminoglycans from lung tissues of germfree (Nos. 1–4) and conventional control (Nos. 5–8) dogs. Two hundred fifty to three hundred microgram uronate material was used for each chromatogram. Absorbance units are not indicated in the figure because all the chromatograms are shown together. Each point on the absorbance scale is 0.1 unit. Abbreviations—same as in Table I.

synthesis or decreased catabolism in the germfree state is not known. In conventional animals, due to their exposure to environmental factors like microorganisms and toxins, it is possible that the rate of degradation of GAG is higher than in germfree animals.

TABLE II. ANALYSES OF HEPARAN SULFATE FRACTIONS FROM GERMFREE AND CONVENTIONAL DOG LUNGS

Group	Animal No.	UA[a] (GlcUA:IdUA)	Total sulfate[b]	N-Sulfate[b]	N-Acetyl groups[b]
Germfree	1	0.98 (86:14)	1.02	0.20	0.74
	2	0.84 (90:10)	0.98	0.15	0.81
	3	0.97 (91:9)	0.88	0.18	0.86
	4	1.07 (92:8)	1.06	0.16	0.79
	Mean	0.97	0.99	0.17	0.80
	SE	0.05	0.04	0.01	0.03
Conventional	5	1.12 (76:24)	1.08	0.19	0.73
	6	0.89 (80:20)	1.10	0.19	0.84
	7	0.97 (85:15)	1.20	0.24	0.70
	8	1.04 (82:18)	1.17	0.21	0.76
	Mean	1.01	1.14	0.21	0.76
	SE	0.05	0.03	0.01	0.03
Significance ($P<$)		NS	0.025	NS	NS

[a] Abbreviations: GlcUA—glucuronic acid; IdUA—iduronic acid; other abbreviations are the same as in Table I.
[b] Mole per mole of hexosamine.

Enzymes that can degrade GAG have been isolated from several microorganisms. Inhalation ot toxins that are present in the environment releases glycohydrolases from alveolar macrophages (20–22), and these enzymes may also be responsible for degradation of GAG in conventional dogs.

The findings also indicate that variations exist in the relative proportions of individual GAG between germfree and conventional dog lungs. Germfree dog lungs had significantly greater proportions of hyaluronic acid and chondroitin 4-sulfate. Although the difference in concentration of chondroitin 6-sulfate is not statistically significant, the mean value in conventional dogs is about 6% less than in germfree dogs. These three GAG are susceptible to hyaluronidase. The enzyme occurs in microorganisms and in certain mammalian tissues. The observations suggest that the activity of hyaluronidase or hyaluronidase-like enzymes might be less in germfree dog lungs than in conventional dog lungs. The higher activity of the enzyme might have resulted in a lower concentration of hyaluronic acid and chondroitin sulfates in conventional dogs.

The results also indicate that there were differences between the two groups in the concentration of hyaluronidase-resistant GAG, heparin, heparan sulfate, and dermatan sulfate. The three GAG have certain common features: all of them are sulfated, contain L-iduronic acid as one of the constituent sugars, and in addition, heparin and heparan sulfate contain N-sulfated glucosamine. These three GAG are hydrolyzed by α-iduronidase. The higher concentration of these GAG in conventional dog lungs could be due to greater biosynthesis of one or more of these constituents or lower activity of α-iduronidase in the tissue. The differences in the contents of iduronic acid and total sulfate in heparan sulfate fractions between the groups suggest the possibility of greater synthesis of these constituents in conventional animals rather than a lower activity of α-iduronidase. Furthermore, variations in the activity of α-iduronidase may result in differences in the total content of heparan sulfate fractions but not difference in their composition. Iduronic acid is formed from glucuronic acid residue of oligosaccharide units of GAG through C-5 epimerization (23, 24). Sulfation occurs through 3′-phosphoadenosine-5′-phosphosulfate (PAPS) by a sulfotransferase (25). Since most of the iduronic acid residues in heparan sulfate are sulfated, any increase in iduronic acid content in the new heparan sulfate may reflect in total sulfate content. It is therefore hypothesized that the conventional animals, like germfree animals, have the same activity of sulfotransferase but increased activity of D-glucuronosyl 5-epimerase and the hypothesis needs to be tested.

Although the dogs were raised under germfree conditions, the role of other environmental factors that could influence the GAG composition cannot be ruled out.

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