J. Biochem. **119**, 1189-1195 (1996)

Developmental Changes of Neutral Glycosphingolipids as Receptors for Pulmonary Surfactant Protein SP-A in the Alveolar Epithelium of Murine Lung[1]

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Received for publication, February 21, 1996

A dramatic change in the glycosphingolipid composition in murine lung occurred between 1 day and 1 week after birth. GlcCer and LacCer were the predominant neutral glycosphingolipids prenatally and 1 day after birth, and the concentrations of globo- and ganglio-series glycosphingolipids increased abruptly from 1 week after birth, reaching maxima at 2-3 weeks. To explore the functional significance of the change, we examined the role of neutral glycosphingolipids as receptors for the murine pulmonary surfactant protein, SP-A, and found that SP-A bound to LacCer, Gg_3Cer, and Gg_4Cer, but not to Gb_3Cer, Gb_4Cer, $IV^3GalNAc\alpha$-Gb_4Cer, sulfatide, or several gangliosides. On TLC-blotting with ^{125}I-labeled SP-A, the binding of SP-A to Gg_3Cer and Gg_4Cer was 5 times higher than that to LacCer, and on immunohistochemical staining Gg_4Cer and Gg_3Cer was mainly observed in the alveolar epithelium. Thus, the capacity to retain SP-A of glycolipid receptors per gram dry weight of lung at 1 week after birth was 1.6 times higher than that at 1 day after birth, and reached a maximum 3 weeks after birth. These findings suggest that the enhanced synthesis of the ganglio-series neutral glycosphingolipids 1 week after birth results in an increase in the binding capacity for SP-A on the epithelial cell surface of alveoli.

Key words: blotting assay, c-type lectin, gangio-series, glycolipid, immunohistochemistry.

Glycosphingolipids are hybrid molecules consisting of a hydrophilic carbohydrate chain and a hydrophobic ceramide. Their carbohydrate chains vary among organs, tissues, and cells, and their expression changes in the course of proliferation and differentiation. Some carbohydrate chains are species-specific. They act as receptors for bacteria, viruses, bacterial toxins, and animal lectins, as antigens related to differentiation and transformation, and as mediators for cell-to-cell interaction (*1-4*). Tissue-specific expression of glycosphingolipids and their changes in concentration during development are thought to be important for the construction and functional establishment of individual tissues, though no conclusive evidence of this has been reported yet.

During the development of lung, morphological and biochemical changes lead to establishment of the respiratory function. One of the changes observed just before birth is the formation of a surfactant layer composed of surfactant phospholipids and apoproteins. Reduced secretion of the surfactant phospholipids and/or low levels of the apoproteins frequently cause the neonatal respiratory distress syndrome, indicating the importance of the surfactant layer for establishment of the respiratory function.

A surfactant is a complex substance containing surfactant apoproteins, SP-A, B, C, and D, and phospholipids (*5*). SP-A, which is the major surfactant apoprotein, has a molecular weight of 26-35 kDa, is composed of collagenous and globular domains, exhibits C-type lectin activity, and belongs to the collectin family (*6, 7*). In the presence of phospholipids, SP-B and SP-C, SP-A endows the biophysical properties of a surfactant (*5, 8*), and plays a role in enhanced secretion and recycling of the surfactant by alveolar type II cells (*9, 10*). Also, SP-A binds with influenza virus (*7*), pollen grains (*11*), myosin (*12*), and alveolar macrophages (*13*), indicating its involvement in the host immune defense (*13*). Concerning the lectin domain of SP-A, its Ca^{2+}-dependent binding with Man-, Fuc-, Gal-, and Glc-conjugated bovine serum albumin (*14*), and glycosphingolipids (*15, 16*) has been well characterized (*17*). In particular, glycosphingolipids on the surface of alveoli are thought to be important for spreading of the surfactant, and SP-As prepared from different animal species exhibit distinct carbohydrate binding specificities (*15, 16*). In this study, we first analyzed the developmental changes in the glycosphingolipid composition in murine lung, and then identified glycosphingolipids acting as receptors for SP-A. A characteristic change in the neutral

[1] This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas, No. 05274107, from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: TLC, thin-layer chromatography; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; GVB, gelatin-containing veronal buffer; CRD, carbohydrate recognition domain.

glycosphingolipid composition during the development of murine lung occurred at the period of surfactant spreading on the surface of alveoli.

MATERIALS AND METHODS

Materials—The glycosphingolipids used in this experiment prepared in our laboratory were from the following sources: GlcCer, LacCer, Gb_3Cer, and Gb_4Cer from human erythrocytes, Gg_3Cer from guinea pig erythrocytes, GalCer and $II^3NeuAc\alpha$-Gg_4Cer from human brain, $IV^3GalNAc\alpha$-Gb_4Cer from horse kidney, and Gg_4Cer from $II^3NeuAc\alpha$-Gg_4Cer by treatment with formic acid. Anti-glycosphingolipid antisera were raised in rabbits (New Zealand White) as follows. One milligram of Gg_3Cer, Gg_4Cer, $II^3NeuAc\alpha$-Gg_4Cer or $IV^3GalNAc\alpha$-Gb_4Cer, and 0.5 mg of bovine serum albumin were dissolved in 1 ml of PBS and then mixed well with 1 ml of Freund's complete adjuvant to obtain a stable water-in-oil emulsion. The emulsion was injected intradermally into the footpads of a rabbit. The antibody titer of the serum was monitored by means of an ELISA. When the titer reached the maximum level (about 1:600,000), the animal was bled and the serum was collected. Antisera against individual glycolipids did not cross-react with each other.

Extraction and Separation of Glycosphingolipids from Murine Lung—Mice of various ages (DBA, male; 18 days of gestation, and 1 day and 1-5 weeks after birth) were purchased from Japanese Biological Materials, Tokyo. After anesthesia with diethylether, their lungs were surgically removed, weighed, and lyophilized.

Total lipids were extracted from the lyophilized lung tissue (0.079-0.135 g dry tissue weight) with chloroform/methanol/water (20 : 10 : 1, 10 : 20 : 1, 20 : 10 : 1, and 10 : 20 : 1, v/v/v) at 45°C, and then the total volume of the combined extracts was adjusted to 12 ml. After determination of free cholesterol and lipid-bound phosphorus (*18*), the remaining lipid extracts were directly applied to a DEAE-Sephadex A-25 (acetate form; Pharmacia Fine Chemicals, Uppsala, Sweden) column (5 ml bed volume), and the column was eluted successively with 15 ml of chloroform/methanol (1 : 1, v/v), 5 ml of methanol, and 50 ml of 0.3 M sodium acetate in methanol. The first and second fractions containing neutral glycosphingolipids were evaporated to dryness, and then acetylated with 20 ml of acetic anhydride and 30 ml of pyridine at room temperature overnight. The acetylated glycosphingolipids were purified by Florisil column chromatography (*19*) and then deacetylated with 0.5 M NaOH in methanol at 37°C for 1 h. After neutralization with 1 M acetic acid in methanol, salts were removed with a Sep-Pak C18 cartridge (*20*). On the other hand, the third fraction eluted from the column with 0.3 M sodium acetate in methanol was saponified, as described above, to remove the contaminating acidic phospholipids, desalted by dialysis, and then evaporated to dryness. The residue thus obtained was dissolved in chloroform/methanol (1 : 1, v/v), and then the content of lipid-bound sialic acid in the solution was determined by the resorcinol-HCl method (*21*).

TLC and TLC-Immunostaining with Cholera Toxin, Anti-Glycolipid, and Anti-Lung Surfactant Protein SP-A Antisera—Neutral and acidic glycosphingolipids, corresponding to 2 mg dry tissue weight, were applied on a TLC plate (0.25 mm thick, Kiesel Gel 60; Merck, Darmstadt, Germany), and developed with chloroform/methanol/water (65 : 35 : 8, v/v/v) and chloroform/methanol/0.5% $CaCl_2$ in water (55 : 45 : 10, v/v/v), respectively. The spots were visualized with orcinol-H_2SO_4 reagent for neutral glycosphingolipids and with resorcinol-HCl reagent for gangliosides, and the density of the spots was quantitated with a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto) at 430 nm for orcinol-H_2SO_4 reagent-positive spots, and at 570 nm for resorcinol-HCl reagent-positive spots (*22*). The spots were also detected using a TLC-immunostaining procedure with several specific ligands (*23*), as follows. A plastic TLC plate (Polygram SIL; Macherey-Nagel, Duren, Germany), on which glycosphingolipids had been separated as described above, was blocked with a blocking buffer (1% polyvinylpyrrolidone, 1% ovalbumin, and 0.02% NaN_3 in phosphate-buffered saline), and then incubated with either rabbit anti-II^3NeuAc-Gg_4Cer (1:500), anti-$IV^3GalNAc\alpha$-Gb_4Cer (1:2,000), anti-Gg_4Cer (1:1,000), or anti-Gg_3Cer (1:500) antiserum, followed by with horseradish peroxidase-conjugated anti-rabbit Ig(G+M+A) antibodies (1:500). For the detection of II^3NeuAc-Gg_4Cer, cholera toxin (1 pg/ ml; Sigma, St. Louis, MO, USA), rabbit anti-cholera toxin antiserum (1:500), and peroxidase-conjugated anti-rabbit Ig(G+M+A) antibodies (1:500) were also used. The antibodies remaining on the plate were detected by reaction with 4-chloro-1-naphthol and H_2O_2 in 50 mM Tris-HCl (pH 7.4) containing 200 mM NaCl at 37°C for 10 min, and then the density of the spots was quantitated with a TLC-densitometer at 570 nm. The binding of SP-A to glycolipids was also analyzed by TLC-immunostaining with a murine lung lavage solution (170 μg/ml protein) and rabbit anti-SP-A antiserum (1:500).

Immunohistochemical Staining of Murine Lung with Anti-Glycolipid Antisera—The indirect immunohistochemical staining of murine lungs by the fluorescent antibody method at various stages of development was performed with anti-Gg_4Cer and anti-Gg_3Cer antisera as described previously (*23*). Tissues embedded in an OCT-compound (Miles Lab., Naperville, IL, USA) were frozen on a dry ice, and then cut into 4-μm-thick sections. The sections were fixed in cold acetone, treated with 0.3% H_2O_2 in methanol to remove endogenous peroxidase, and then incubated with anti-glycosphingolipid antiserum diluted 1:200 for 2 h at room temperature in a humidified chamber. Next, they were incubated with FITC-labeled anti-rabbit Ig(G+A+M) antibodies diluted 1:50 for 30 min at room temperature. The sections were finally washed with PBS, and then examined under a fluorescence microscope (incident illumination type).

Purification and Radioiodination of Pulmonary Surfactant Protein SP-A—Mice (DBA) were injected intratracheally with silica (100 mg of silica/kg body weight), and after 28 days, their lungs were washed three times with 5 mM hepes buffer (pH 7.4) containing 0.15 M NaCl and 1 mM phenylmethylsulfonylfluoride (PMSF) (*24, 25*). The washings from 100 mice were pooled and centrifuged at $800 \times g$ for 10 min to remove cells. Then the solution, to which $CaCl_2$ had been added to a concentration of 5 mM, was centrifuged at $40,000 \times g$ overnight, and the resultant pellet was dispersed in 5 mM hepes buffer (pH 7.4) containing 1.64 M NaBr, 0.15 M NaCl, and 1 mM PMSF with the

aid of sonication. The solution was then centrifuged at 100,000 × *g* and this step was repeated once more. Then, the pellet was resuspended in distilled water and extracted with *n*-butanol at room temperature (*26*). The delipidated pellet, which contained the pulmonary surfactant apoproteins, was collected by centrifugation at 1,500 × *g* for 30 min, suspended in 5 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$, and then dialyzed against the same buffer at 4°C. The solution, after removal of the insoluble material by centrifugation at 100,000 × *g* for 1 h, was applied on a mannose-conjugated Sepharose 6B column, and SP-A was eluted from the column with a gradient from 0 to 2 mM EDTA in 5 mM Tris-HCl (pH 7.4) (*14*). The SP-A, which was eluted from the column with about 1.5 mM EDTA in Tris-HCl, was further purified by gel filtration on BioGel A5m and analyzed by SDS-polyacrylamide gel electrophoresis. The purified SP-A was radiolabeled with a radioiodination system with lactoperoxidase and glucose-fixed beads (NEN) (*27*), and the specific activity was determined to be 1.4 μCi/μg. The radioiodinated SP-A was utilized for the analysis of binding to glycosphingolipids on a TLC plate as described above. The protein concentration was determined by the method of Lowry *et al.* with bovine serum albumin as the standard (*28*).

Preparation of Antiserum against Pulmonary Surfactant Protein SP-A—Antiserum against murine SP-A was raised by immunizing a rabbit (NZW) with the purified SP-A (100 μg) and Freund's complete adjuvant, a booster immunization being performed 10 days after the initial injection of antigen. The antibody titer was monitored by ELISA, and serum with a sufficiently high titer was collected 3 weeks after the last immunization. Prior to use, the antiserum was absorbed by affinity chromatography with murine serum proteins bound to CNBr-activated Sepharose 4B (Pharmacia), and was found to react with the purified SP-A by SDS-PAGE and Western blotting. Control serum was obtained from a nonimmunized rabbit.

Electrophoresis and Western Immunoblotting—SDS-PAGE, followed by Western blotting on a nitrocellulose sheet with anti-SP-A antiserum, was performed according to the method of Towbin *et al.* (*29*). A nitrocellulose sheet, to which proteins in the lung lavage solution had been electrotransferred from the SDS-polyacrylamide gel, was treated with 0.5% BSA in PBS to block nonspecific binding, and then subsequently reacted with rabbit anti-SP-A antibodies, and with horseradish peroxidase-conjugated goat anti-rabbit Ig(G+A+M) antibodies. The antibodies remaining on the sheet were detected using an Immunostain kit (Konica, Tokyo).

Binding Analysis of Murine Pulmonary Surfactant Protein SP-A to Glycolipids by ELISA and SDS-PAGE—Binding of SP-A to a polystyrene microtiter plate (96 wells; Becton Dickinson, San Jose, CA, USA) coated with several glycolipids, followed by ELISA and SDS-PAGE of the bound proteins, was performed as follows. Glycosphingolipids, GlcCer, LacCer, Gb_3Cer, Gb_4Cer, Gg_3Cer, Gg_4Cer, $II^3NeuAc\alpha$-Gg_4Cer, and $IV^3GalNAc\alpha$-Gb_4Cer (200 μg/ml in ethanol), were placed in each well of the ELISA plate and the solvent was evaporated off. After blocking each well with a blocking buffer (1% BSA in GVB) and washing with a washing buffer (0.1% BSA in GVB), 200 μl of a murine lung lavage solution, containing 170 μg/ ml of protein, was added to each well, followed by incubation at 37°C for 2 h. The amount of SP-A bound to the ELISA plate was measured by ELISA with anti-SP-A antiserum and horseradish peroxidase-conjugated anti-rabbit Ig(G+A+M) antibodies. In a separate experiment, the proteins remaining on the ELISA plate after washing 3 times with the washing buffer were recovered with 20 μl of the sample buffer (10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.125% bromophenol blue in 0.125 M Tris-HCl, pH 6.8) at 80°C for 30 min. The proteins in the surpernatant and the sample buffer were analyzed by SDS-PAGE.

RESULTS

Developmental Changes in Neutral and Acidic Glycosphingolipids in Murine Lung—As shown in Fig. 1, the major neutral glycosphingolipids in murine lung on the 18th day of gestation and 1 day after birth were GlcCer and LacCer, amounting to about 96% of the total neutral glycosphingolipids (Table I). At 1 and 3 weeks after birth, the concentrations of neutral glycosphingolipids with longer carbohydrate chains were dramatically increased, and,

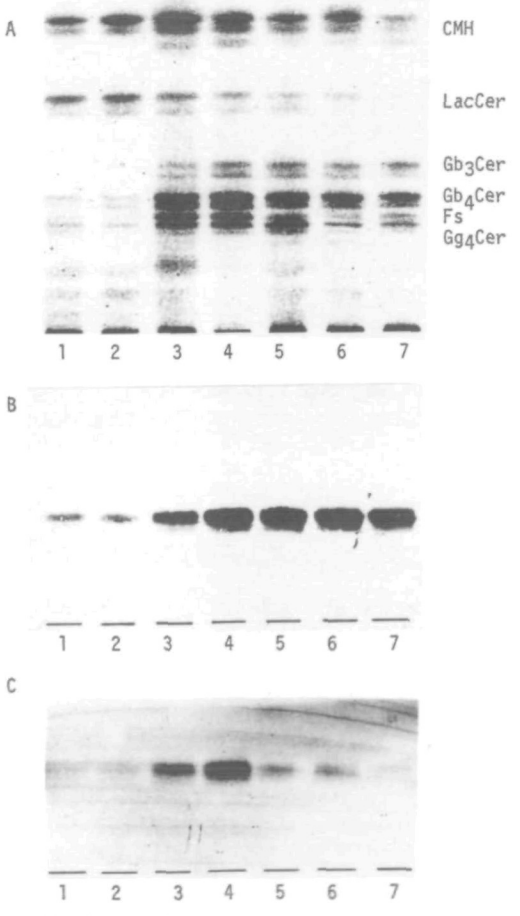

Fig. 1. **TLC of neutral glycosphingolipids in the lungs of DBA mice at different stages of development.** Neutral glycosphingolipids, corresponding to 2 mg (A) and 0.5 mg (B, C) dry tissue weight, were developed on a TLC plate with chloroform/methanol/water (65 : 35 : 8), and detected with orcinol-H_2SO_4 reagent (A), anti-Gg_4-Cer antiserum (B), or anti-$IV^3GalNAc\alpha$-Gb_4Cer antiserum (C). Lanes: 1, 18 days gestation; 2, 1 day after birth; 3, 1 week after birth; 4, 2 weeks after birth; 5, 3 weeks after birth; 6, 4 weeks after birth; 7, 5 weeks after birth.

concomitantly, LacCer decreased to one-third. The neutral glycosphingolipids with longer carbohydrate chains were characterized by TLC-immunostaining with specific antibodies against Gg_4Cer, Gg_3Cer, and $IV^3GalNAc\alpha$-Gb_4Cer, and their structures were concluded to be Gg_4Cer, Gg_3Cer, and $IV^3GalNAc\alpha$-Gb_4Cer (Fig. 1, B and C). The concentrations of $IV^3GalNAc\alpha$-Gb_4Cer and Gg_4Cer at 1 week were 11 and 9 times higher than those at 1 day, respectively, and continuously increased up to 2 weeks. Thereafter, the rate of reduction in the concentration of $IV^3GalNAc\alpha$-Gb_4Cer was higher than that of Gg_4Cer. Neutral glycosphingolipids other than GlcCer and LacCer, such as Gb_3Cer, Gg_3Cer, Gb_4Cer, $IV^3GalNAc\alpha$-Gb_4Cer, and Gg_4Cer, comprised 37% of the total neutral glycosphingolipids in murine lung at 2 weeks. At 5 weeks, neutral glycosphingolipids with more than 3 carbohydrate residues comprised 50% of the total neutral glycosphingolipids, and LacCer 4.8%. On the other hand, the major acidic glycosphingolipid in murine lung was GM3, whose concentration in lung after 1 week was the highest in the total glycosphingolipid fraction, and its developmental change was similar to that of Gb_4Cer (Fig. 2A). GM1 was detected on TLC-immunostaining with cholera toxin, but its concentration did not change during development (Fig. 2B).

Immunohistochemical Localization of Gg_4Cer and Gg_3Cer in Murine Lung during Development—Gg_4Cer and Gg_3Cer vary significantly during the development of murine lung, and their localization in the lung on the 18th day of gestation and at 7 weeks after birth was analyzed by indirect immunohistochemical staining. As shown in Fig. 3, Gg_4Cer at 18 days gestation was found to be distributed in the alveolar epithelium, particularly in the epithelial cells. Its distribution in lung at 7 weeks after birth was essentially the same as that at 18 days gestation, although its concentration was significantly increased. The same pattern was obtained on staining with anti-Gg_3Cer antibodies (data not shown).

Interaction of Pulmonary Surfactant Protein SP-A with Glycosphingolipids—SP-A purified from the lung lavage solution had an estimated molecular weight of 30 kDa, and was used for the preparation of antiserum and radioiodinated SP-A (Fig. 4). A TLC-blotting experiment with the radioiodinated SP-A, as shown in Fig. 5, indicated that

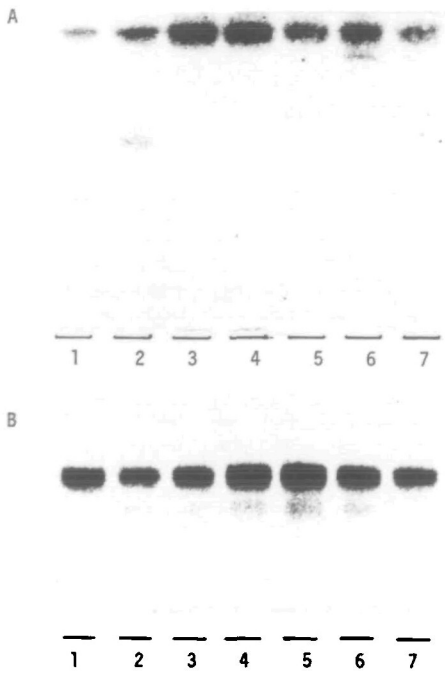

Fig. 2. **TLC of acidic glycosphingolipids in the lungs of DBA mice at different stages of development.** Acidic glycosphingolipids, corresponding to 2 mg (A) and 0.1 mg (B) dry tissue weight, were developed on a TLC plate with chloroform/methanol/water (55 : 45 : 10), and detected with resorcinol-HCl reagent (A) or cholera toxin (B). The samples are the same as in Fig. 1.

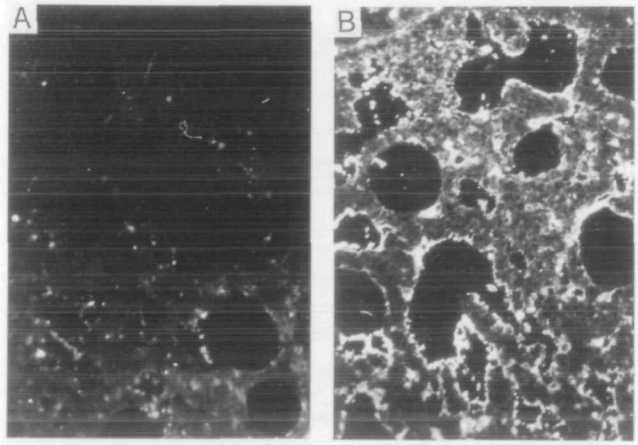

Fig. 3. **Immunohistochemical localization of Gg_4Cer in murine lung on the 18th day of gestation.** The tissue sections were incubated with rabbit anti-Gg_4Cer antiserum (B) or control rabbit serum (A), followed by with FITC-conjugated anti-rabbit Ig(G + A + M) antibodies, as described in the text.

TABLE I. **Concentrations of cholesterol, lipid-bound phosphorus, and neutral and acidic glycosphingolipids in murine lung at different stages of development.**[a]

Stage	Cholesterol	Lipid-bound phosphorus	CMH	LacCer	Gb_3Cer	Gg_3Cer	Gb_4Cer	Gg_4Cer	Fs	GM3	GM1
	(μmol/g dry weight)		(nmol/g dry weight)								
18 days gestation	26.8	57.8	213.5	177.6	2.3	tr	5.1	4.1	3.2	96.6	3.1
1 day	37.4	58.6	262.2	171.5	2.4	tr	5.7	4.2	3.5	118.2	6.3
1 week	30.3	65.1	350.0	48.1	17.5	11.2	65.3	40.7	39.4	421.7	5.3
2 weeks	35.4	64.9	397.2	44.1	20.5	25.3	88.0	66.0	63.8	477.4	6.0
3 weeks	57.6	85.5	276.0	59.4	29.1	31.7	73.5	69.8	14.4	374.1	6.2
4 weeks	56.6	71.4	217.7	22.7	23.3	28.3	68.2	54.6	17.5	330.1	6.6
5 weeks	60.2	78.2	139.8	14.7	21.7	22.9	65.6	39.8	3.0	372.0	5.2

[a]tr, trace amount. Mean values for three pooled tissue samples are shown; the standard deviations were within 10%. Fs, Forssman antigen.

SP-A bound to LacCer, Gg_3Cer, and Gg_4Cer, but not to GlcCer, Gb_3Cer, Gb_4Cer, or Forssman antigen ($IV^3GalNAc\alpha$-Gb_4Cer) among the neutral glycosphingolipids detected with orcinol-H_2SO_4 reagent in murine lung. Quantitative analysis by TLC-blotting with known amounts of purified glycolipids indicated that Gg_3Cer and Gg_4Cer were effective receptors for SP-A, Gg_3Cer being slightly better than Gg_4Cer, and LacCer bound only one-fifth of SP-A on Gg_4Cer (Fig. 6). In addition, SP-A was not observed to bind with globo- or lacto-series neutral glycolipids, I^3SO_4-GalCer, or gangliosides, such as GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b, and fucosyl GM1. Thus, it is concluded that the affinity of glycolipid-receptors for SP-A on the pulmonary epithelium was significantly enhanced 1 week after birth by expression of Gg_3Cer and Gg_4Cer. The overall capacity to retain SP-A of glycolipid-receptors per gram dry weight of lung at 1 week after birth was 1.6 times higher than that at 1 day, and reached the maximum at 3 weeks after birth (Fig. 7). The same binding specificity of SP-A was observed on TLC-immunostaining with a lung lavage solution and rabbit anti-SP-A antiserum. Gg_3Cer from guinea pig erythrocytes and Gg_4Cer from human brain gangliosides were shown to be effective receptors for SP-A (data not shown), indicating that the binding specificity of SP-A is not modified by the radiolabeling procedure. In addition, SDS-PAGE of the lung lavage solution after reaction with several glycolipids coated on an ELISA plate

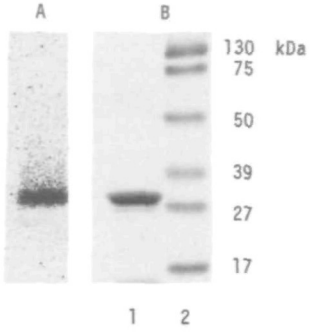

Fig. 4. **SDS-polyacrylamide gel electrophoresis (B) of murine pulmonary surfactant protein SP-A (1) purified from a lung lavage solution of DBA mice, and a radioautogram of radioiodinated SP-A (A).** The gel (B) was stained with Coomassie Brilliant Blue R-250. 2, standard protein mixture (Pre-stained proteins, Bio-Rad): 130 kDa, phosphorylase *b*; 75 kDa, bovine serum albumin; 50 kDa, ovalbumin; 39 kDa, carbonic anhydrase; 27 kDa, soybean trypsin inhibitor; 17 kDa, lysozyme.

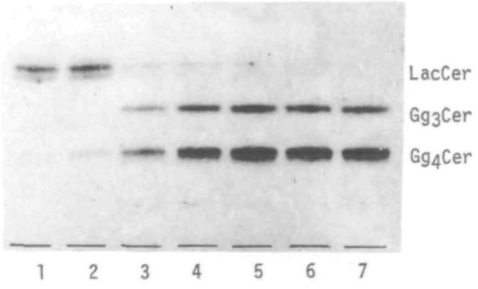

Fig. 5. **TLC-blotting analysis with radioiodinated SP-A of neutral glycosphingolipids in the lungs of DBA mice at different stages of development.** Neutral glycosphingolipids, corresponding to 2 mg dry tissue weight, were developed on a TLC plate as described in the legend to Fig. 1, and then reacted with ^{125}I-labeled SP-A.

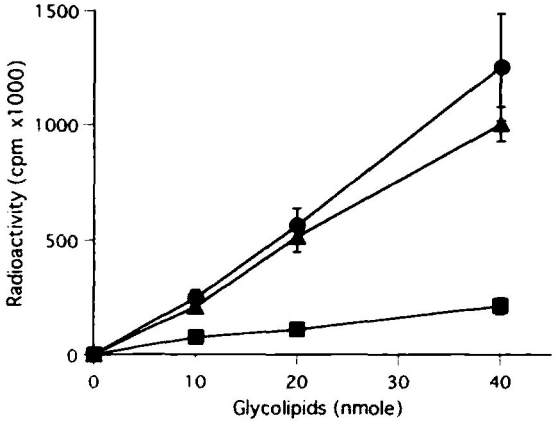

Fig. 6. **Reactivities of LacCer (■), Gg_3Cer (●), and Gg_4Cer (▲) after reaction with radioiodinated SP-A on TLC-blotting.** The levels of radioactivity detected for the globo-series glycolipids (Gb_3Cer, Gb_4Cer, and Forssman antigen), GalCer, sulfatide, and gangliosides were below the background level.

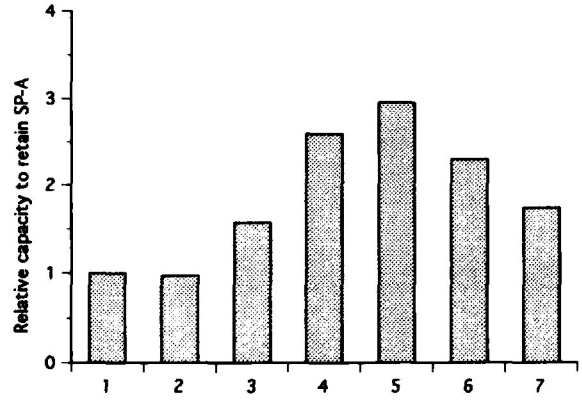

Fig. 7. **Developmental change in the capacity to retain surfactant SP-A of glycolipid-receptors expressed per gram dry weight of lung.** The relative amounts of SP-A, which was expected to be retained by glycolipid-receptors, were compared with that on the 18th day of gestation (= 1.00). Columns: 1, 18 days gestation; 2, 1 day after birth; 3, 1 week after birth; 4, 2 weeks after birth; 5, 3 weeks after birth; 6, 4 weeks after birth; 7, 5 weeks after birth.

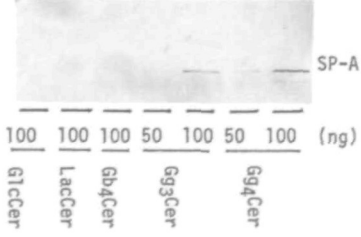

Fig. 8. **Western blotting of proteins bound to glycosphingolipids in a murine lung lavage solution.** After reaction of a murine lung lavage solution with glycosphingolipids (50 and 100 ng) coated on an ELISA plate, the proteins bound on the plate were recovered in the sample buffer [10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.125% bromophenolblue in 0.125 M Tris-HCl (pH 8.6)], and analyzed by Western blotting with anti-SP-A antiserum.

TABLE II. **Concentration of pulmonary surfactant protein SP-A in murine lung as determined by SDS-PAGE.** Homogenates of murine lung were analyzed by SDS-PAGE and Western blotting with anti-SP-A antiserum. The gels, with a known amount of BSA, were stained with Coomassie Brilliant Blue, and the density of the bands corresponding to those positive with anti-SP-A antiserum was measured at 500 nm with a TLC-densitometer.

Stage	30 kDa (μg protein/g wet lung)
18 days gestation	38.0±13.2
1 day	71.0±25.5
1 week	157.5±45.9
2 weeks	420.9±46.2

showed that SP-A was recovered only when the lung lavage solution was incubated on an ELISA plate coated with Gg_4Cer and Gg_3Cer (Fig. 8). No SP-A was recovered from the well coated with LacCer, probably due to the relatively low affinity of LacCer for SP-A, as determined above. Thus, intact murine SP-A in the lung lavage solution was shown to react with the ganglio-series neutral glycosphingolipids, Gg_3Cer and Gg_4Cer, both of which showed an increase in concentration after birth, and were distributed in the alveolar epithelium of murine lung and served as receptors for SP-A.

Relationship between the Concentrations of Pulmonary Surfactant Protein SP-A and Glycolipid-Receptors—On Western blotting with anti-SP-A antiserum, a protein with an estimated molecular weight of 30 kDa was detected in the homogenate of murine lung, and the concentration of this protein was examined densitometrically after staining of the SDS-PAGE gel with Coomassie Brilliant Blue, as shown in Table II. The change in the concentration of this 30 kDa protein was rather coincident with the increased concentrations of Gg_3Cer and Gg_4Cer, indicating that glycolipid-receptors exhibiting high affinity are prepared in association with the increased synthesis of SP-A in the period of alveolar extension.

DISCUSSION

The primary function of pulmonary surfactants is reduction of the surface tension of pulmonary fluid at the alveolar interface. The formation of a tightly packed monolayer of surfactants contributes to the prevention of alveolar collapse during expiration (*30, 31*). The pulmonary surfactants consist of 80% phospholipids and 10% proteins. The major apoprotein, SP-A, binds to the major surfactant phospholipid, dipalmitoylphosphatidylcholine, in the presence of Ca^{2+} (*32*), and plays an essential role in the formation of the monolayer together with SP-B, SP-C, and SP-D (*5*). Its collagen-like domain interacts with macrophages, contributing to the immune defense in the lung (*33*). In addition, SP-A carries a carbohydrate recognition domain (CRD), which binds to glycoconjugates on the alveolar epithelium for anchoring the surfactant to pollen grains, and to influenza viruses to prevent infections (*7, 11*). The structural requirements of the CRD for the binding of carbohydrates have been found to vary with the SP-A of different animals (*34*). By utilizing glycolipids on a TLC plate, the following have been characterized as receptors for SP-A: GalCer, GlcCer, and LacCer for human SP-A (*15*), GalCer, LacCer, and Gg_4Cer for human SP-A (*17*), GalCer for dog SP-A (*15*), trihexaosyl and tetrahexaosyl ceramides for rat SP-A (*15*), and GalCer and Gg_3Cer for rat SP-A (*16*). As reported in this paper, we first examined glycolipid candidates for the receptor of murine SP-A using radioiodinated SP-A, and identified three glycolipids, LacCer, Gg_3Cer, and Gg_4Cer, as receptors. Among them, the affinity of Gg_3Cer and Gg_4Cer for SP-A was greater than that of LacCer. Only Gg_3Cer and Gg_4Cer were detected in the lungs of mice older than 1 week after birth, and a reduction in the LacCer concentration was observed. These results for murine lung were identical to those for rat lung reported by Childs *et al.* (*15*), but slightly different from those for rat lung reported by Kuroki *et al.* (*16*), probably due to the difference in the labeling procedures for SP-A. The expression of Gg_3Cer and Gg_4Cer on the lung alveolar surface 1 week after birth, when the respiratory function is almost fully established, might contribute to maintain the surfactant layer, and the dramatic change in glycosphingolipid composition from 1 day to 1 week might be very important. The concentrations of Gg_3Cer and Gg_4Cer in the lung continued to increase up to 3 weeks after birth. These changes were associated with ones of SP-A, but were different from those of the globo-series glycolipids, Gb_4Cer and Forssman antigen. Since Forssman antigen was detected immunohistochemically in the mesenchymal cells but not in the epithelial cells of the lung, the developmental changes of the globo-series and ganglio-series glycolipids were thought to reflect the differences in the distributions of these glycolipids, *i.e.* globo-series glycolipids are abundant in mesenchymal tissues and ganglio-series glycolipids in epithelial cells (K. Momoeda *et al.*, unpublished results). Similar distributions of Forssman antigen and Gg_4Cer have been described for adult mouse intestine (*35*). It is clearly shown in Fig. 3 that Gg_4Cer is an antigen in the epithelial cells of murine lung. Since the affinity of SP-A with Gg_4Cer and Gg_3Cer was 5 times higher than that with LacCer, the capacity to bind SP-A expressed as glycolipid-receptors per gram dry weight of lung at 3 weeks after birth was 3-fold at 1 day after birth (Fig. 7). Thus, the developmental change in the glycosphingolipid composition in the murine lung seems to be related to the establishment of the respiratory function, and interaction of glycosphingolipids with pulmonary surfactant apoproteins plays a role in the establishment.

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