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A mathematical model to predict protein wash out kinetics during whole-lung lavage in autoimmune pulmonary alveolar proteinosis

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pulmonary alveolar proteinosis; granulocyte/macrophage colony-stimulating factor autoantibody; whole-lung lavage; protein transfer rate

PULMONARY ALVEOLAR PROTEINOSIS (PAP) is a rare lung disorder in which surfactant-associated phospholipids and proteins abnormally accumulate within alveoli and terminal bronchioles, leading to impaired gas exchange and progressive respiratory failure (6, 33, 40). PAP is classified into three groups based on etiology: autoimmune PAP (aPAP), secondary PAP, and hereditary PAP (6, 17, 40). aPAP is caused by granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibodies, which prevent surfactant removal by alveolar macrophages (20, 41). aPAP is the most prevalent form of PAP, comprising 90% of all PAP cases (6, 17, 40). Currently, whole-lung lavage (WLL) remains the only standard therapy for aPAP (4, 7, 29). Although WLL improves PAP in about 85–95% of patients, around 15–66% of such patients may require multiple and repeated WLL therapy (1, 4, 37). Removal of the lipoproteinous material by WLL immediately improves both lung volume and ventilation/perfusion ratio, leading to a marked increase in arterial oxygen gas pressure (5, 29, 36). In contrast, the diffusion capacity recovers gradually and incompletely over a 6-mo period (36). In addition, WLL decreases the area of ground-glass opacities but not reticular opacities and inter-

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lobular septal thickening (24). These observations suggest that the efficacy of WLL is not attributable to simple exclusion of accumulated surfactants but rather attributable to the recovery of normal lung structure and function.

GM-CSF autoantibodies and various other proteins (with the exception of large molecules such as IgM) have been reported to transfer the air-blood barrier (2, 9, 39). Both IgG1/albumin and IgG2/albumin ratios of the serum and bronchoalveolar lavage fluid (BALF) are similar, indicating transfer of these proteins (28). IgG most probably migrates by epithelial transcytosis or by paracellular diffusion through the air-blood barrier (13). In the steady state, the air-blood barrier consists of endothelial cells, basement membrane, epithelial cells, and surfactant film (8, 15). Surfactant film reduces leakage of plasma proteins to a minimum (15). In a previous study, disruption of surface tension-lowering properties of surfactant protein B (SP-B) in conditional knockout mice led to constriction of alveolar capillaries that resulted in protein leaks, lung edema, and alterations in alveolar surface area (15). Although no report describes the disruption of air-blood barrier after lung lavage, it is plausible that WLL removes the surfactant film from the alveolar surface followed by leaking plasma proteins.

In the present study, the kinetics of transfer of proteins from the blood and the surfactant to the lavage fluid was examined by measuring their concentrations in aliquots of lavage fluid drained during WLL. For this purpose, we proposed a mathematical model that can account for the transfer of proteins from the blood and the surfactant to the lavage fluid. The transmission coefficients were optimized, and the temporal variations of protein concentrations were simulated. Finally, the proposed model was evaluated by comparison with the measured data. Moreover, we showed the limitations of the present model.

Glossary

A_b	The effective surface area from the blood
A_s	The effective surface area from the surfactant
K_b	The transmission coefficient from the blood
K_s	The transmission coefficient from the surfactant
m_b	The masses of protein in the blood
$m_{in\text{-}out}$	The masses of protein in instilling saline and draining lavage fluid; actually, no protein exists in instilling saline
m_l	The masses of protein in the lavage fluid
m_{out}	The protein mass of drainage
m_s	The masses of protein in the surfactant
R_{cl}	The absorption rate of fluid into the circulation
S_A	The alveolar surface area
V_A	The alveolar volume
V_b	The volume of blood
V_{in}	The fluid volume of instilled saline
V_l	The volume of lavage
$V_{l\text{-}b}$	The fluid volume absorbed into the circulation
V_{out}	The fluid volume of drainage
V_s	The volume of surfactant

MATERIALS AND METHODS

Participants

Nine patients were enrolled in five hospitals in Japan. These hospitals included Tohoku University Hospital, Tokyo Medical University Hachioji Hospital, Aichi Medical University Hospital, Dokkyo Medical University Koshigaya Hospital, and Niigata University Medical and Dental Hospital. Diagnosis of aPAP was performed on the basis of cytological analysis of BALF, pulmonary histopathological findings, or both with high-resolution computed tomography appearance (40). All cases were confirmed to have elevated serum GM-CSF autoantibody levels (21, 41). The institutional review board of each hospital approved the study, and all subjects provided written informed consent. The study protocol was designed according to The Ethical Guideline of Clinical Research by The Japanese Ministry of Health, Labour, and Welfare in 2008.

Data of arterial blood gas analyses and serum markers were collected within 3 days, and pulmonary function tests were within 2 wk prior to WLL.

Procedure of WLL

Seventeen lungs from nine patients with aPAP underwent WLL. We allowed each participating hospital to conduct WLL in accordance with their own procedures. Generally, after administration of general anesthesia, patients were intubated with a double-lumen endotracheal tube to isolate the lungs, after which mechanical ventilation was initiated. After ventilation of the bilateral lungs with 100% oxygen for 5–15 min, saline was instilled into the lavage lung while ventilation of the other lung with 100% oxygen was continued. The instilled saline was then retained for a few minutes and then discharged by gravity into a container until a decrease in outflow was observed. These procedures were then repeated. In each lavage cycle, we prepared a timetable to record the exact time (to the second) of the start of instilling saline, the start of retaining, and the start and end of lavage fluid draining. We measured the volume of drained lavage fluid and used a 10-ml aliquot for further analyses. All samples were stored at −80°C until use.

Measurement of Substance Concentration

The serum and BALF concentration of IgG, GM-CSF autoantibody, transferrin, albumin, β_2-microglobulin, urea, gastrin, and SP-D were measured; IgG were quantified by an ELISA system using Human IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, AL) according to the manufacturer's instructions. GM-CSF autoantibody concentrations were measured by an ELISA system as described previously (17). β_2-Microglobulin, gastrin, urea, and SP-D concentrations were measured by latex agglutination immunoassay (LA; LZ test Eiken β_2-M-II; Eiken, Tokyo Japan), radioimmunoassay (gastrin RIA kit II; Fujirebio, Tokyo, Japan), urease-indophenol method (urea nitrogen test; Wako, Tokyo, Japan), and enzyme immunoassay (SP-D kit Yamasa EIA II; Yamasa, Tokyo, Japan), respectively. Serum transferrin and albumin concentrations were measured by turbidimetric immunoassay (TIA; N-Assay TIA Tf-H Nittobo; Nittobo, Tokyo, Japan) and bromocresol purple dye-binding assay (PureAuto A ALB; Kainos, Toyko, Japan), respectively, and those in the BALF were analyzed by LA (N-Assay LA Micro Tf Nittobo) and TIA (AutoWako Microalbumin). These serum samples were collected just before the beginning of WLL.

A Mathematical Kinetic Model to Estimate the Concentration of Proteins in the Lavage Fluid

We postulated that proteins both in the accumulated surfactant material and in the pulmonary capillaries transfer into the lavage fluid. Under such circumstances, the rate of protein transfer to the lavage fluid is assumed to be as follows:

$$\frac{dm_l}{dt} = \frac{dm_s}{dt} + \frac{dm_b}{dt} + \frac{dm_{in-out}}{dt} \quad (1)$$

where the first term in the right-hand side is the transfer rate from surfactant, the second term is the transfer rate from blood, and the

third term is the rate of instilling and drainage. The transfer rate from surfactant dm_s/dt and the transfer rate from blood dm_b/dt are modeled by analogy to the heat transmission model as

$$\frac{dm_s}{dt} = K_S \cdot A_S\left(\frac{m_s}{V_s} - \frac{m_1}{V_1}\right) \quad (2)$$

$$\frac{dm_b}{dt} = K_b \cdot A_b\left(\frac{m_b}{V_b} - \frac{m_1}{V_1}\right) \quad (3)$$

where K_s and K_b are the transmission coefficient from the surfactant and the blood to the lavage fluid, respectively. A_s and A_b are the effective surface area from the surfactant and the blood to the lavage fluid, respectively. The parameters m_l, m_s, and m_b represent the masses of protein in the lavage fluid, surfactant, and blood, respectively. V_l represents the fluid volume in lavage. V_s and V_b represent the fluid volume in surfactant and blood. We assumed that m_b, V_s, and V_b are constant during the WLL.

We calculated the temporal variation of the mass of protein and the volume of fluid in the stages of instilling, retaining, draining, and preparing in each lavage cycle, as described as follows.

Instilling stage. The volume change of protein and lavage fluid in the lung is expressed as:

$$\frac{dm_{in-out}}{dt} = 0 \quad (4)$$

$$\frac{dV_l}{dt} = \frac{dV_{in}}{dt} - \frac{dV_{l-b}}{dt} \quad (5)$$

where V_{in} is the fluid volume of instilled saline, and $V_{l\text{-}b}$ is the fluid volume absorbed into the circulation expressed as

$$\frac{dV_{l-b}}{dt} = A_b \cdot R_{cl} \quad (6)$$

where R_{cl} is the absorption rate of fluid into the circulation. The concentration of protein was calculated as the ratio of the mass of protein to the fluid volume calculated from *Eqs. 1–6* according to the procedures described in the following subsection.

Retaining stage. No saline is instilling in the retaining stage, which means

$$\frac{dV_{in}}{dt} = 0 \quad (7)$$

The variation of the mass of protein and the volume of fluid were calculated from *Eqs. 1–7*.

Draining stage. The lavage fluid is drained in this stage, which means

$$\frac{dm_{in-out}}{dt} = -\frac{dm_{out}}{dt} \quad (8)$$

$$\frac{dV_l}{dt} = -\frac{dV_{out}}{dt} - \frac{dV_{l-b}}{dt} \quad (9)$$

where m_{out} and V_{out} are the protein mass and the fluid volume of drainage. The variation of the mass of protein and the volume of fluid were calculated from *Eqs. 1–3* and *6*, *8*, and *9*.

Preparing stage. Substance transfer in this stage may be considered to be similar to that in the retaining stage.

Data Processing and Statistics

Data including patient identity, protein concentrations in the serum or in the BALF of the right and left lungs, vital capacity, the number of cycles, the volume of instilled saline or drained lavage fluid, and time for each lavage stage were entered into a file (Microsoft Excel 2010). Using theoretical equations that solved protein concentrations in the drained lavage fluid (described in RESULTS), we wrote a program using Visual Basic Application to calculate the theoretical concentrations of proteins on the basis of specific variables.

Estimation of the protein concentration in the drained lavage fluid was carried out by numerically integrating differential equations using the following parameters: the volume of instilled saline, drained lavage fluid, time of each stage, the concentration of proteins in the first lavage cycle, effective alveolar and capillary surface area described below, and a given set of transmission coefficients, K_s and K_b. The resulting concentration curve was optimized with actual measurements manually by changing transmission coefficients. The effective areas of alveolar surface and pulmonary capillaries were calculated according to the equations described in Appendix A (10).

Numerical data were evaluated for normal distribution by using Shapiro-Wilk tests. Nonparametric data were analyzed by using Kruskal-Wallis rank sum test. Multiple comparisons were performed through a Bonferroni-adjusted Wilcoxon rank-sum test. All tests were two-sided, and P values <0.05 were considered statistically significant. Data were analyzed by using R-version 2.15.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Demographic and Clinical Findings for Study Subjects

Nine patients with active aPAP were enrolled in this study. Demographic data are shown in Table 1.

The mean age at WLL was 54.3 ± 11.4 yr old, with a male-to-female ratio of 2:1. The duration of the disease from onset was variable, ranging 10–96 mo. Patients showed no evidence of active pulmonary infection. Pulmonary functions and laboratory findings are described in Table 2.

The mean arterial oxygen pressure at room air was 64.0 ± 15.4 mmHg in seven patients and 55.2 and 67.4 mmHg for two patients under nasal oxygen supply. Percentage of vital capacity and percentage of carbon monoxide diffusing capacity were moderately to severely suppressed with 72.1 ± 17.7% and 51.0 ± 21.5%, respectively, whereas forced expiratory volume in 1 s/forced vital capacity was relatively conserved with 85.8 ± 10.9%. The mean serum biomarker levels of Krebs von den Lungen-6, SP-D, and carcinoembryonic antigen were 20,720 ± 13,953 IU/ml, 471 ± 271 ng/ml, and 26.7 ± 22.9 ng/ml, respectively. The mean serum GM-CSF autoantibody levels were 45.8 ± 51.7 μg/ml. These patient characteristics were similar to a past large Japanese cohort with PAP (17).

Timetables and Volume Balance for WLL

As shown in Fig. 1, *A* and *B*, each lavage cycle consisted of four stages: instilling (from the beginning to the end of saline instillation), retaining (from the end of saline instillation until the beginning of drainage), draining (from the beginning until the end of drainage), and preparing (from the end of drainage until the beginning of the next saline instillation). Twelve lungs from seven patients underwent WLL with short-term cycles (210–285 s), whereas five lungs from three patients underwent WLL with long-term cycles (550–634 s) (Table 3). In eight patients, both lungs underwent WLL; however, for one patient, only the right lung underwent lavage. Data for instilled saline volume, discharged lavage fluid volume, and time for each of the stages as defined above are shown in Tables 3 and 4. Lavage was repeated 11 to 29 times (median of 20 cycles) until the lavage fluid appeared clearer (Fig. 1*C*).

Time required for total WLL time ranged from 5,200 to 11,796 s. Instilling, retaining, draining, and preparing time

Table 1. *Demographic data on study subjects who underwent WLL*

Case	Age, yr	Sex	Symptoms*	Onset to WLL†, mo	DSS‡	Smoking Status	Occupational Dust Exposure	Complications
1	45	M	DOE, Occasional Cough	66	2	Ex-Smoker	No	Psoriasis, HT, DL
2	54	F	DOE	43	2	Never	No	HT, DL
3	67	M	Dyspnea, Cough, Hemosputum	10	5	Ex-Smoker	No	Postcerebral Infarction
4	34	M	Dyspnea	42	5	Current Smoker	No	None
5	53	F	Dyspnea, Cough	96	5	Never	No	SSS
6	46	M	None	30	1	Current Smoker	Yes	None
7	66	M	DOE	25	4	Never	No	DM
8	67	F	Dyspnea	36	5	Never	No	None
9	57	M	DOE	28	2	Never	No	None

*Symptoms were recognized as respiratory symptoms. †Onset: time when the 1st respiratory symptom emerged or time of finding an abnormal image that was compatible with pulmonary alveolar proteinosis (PAP). ‡Disease severity score (DSS): defined based on respiratory symptoms and arterial oxygen tension (PaO_2; see Ref. 3). DSS 1: no symptoms and $PaO_2 \geq 70$ mmHg. DSS 2: symptomatic and $PaO_2 \geq 70$ mmHg. DSS 3: 60 mmHg $\leq PaO_2 < 70$ mmHg. DSS 4: 50 mmHg $\leq PaO_2 < 60$ mmHg. DSS 5: $PaO_2 < 50$ mmHg. WLL, whole-lung lavage; DOE, dyspnea on exertion; HT, hypertension; DL, dyslipidemia; SSS, sick sinus syndrome; DM, diabetes mellitus.

ranged 10–225 s, 120–425 s, 52–225 s, and 0–96 s, respectively. In 11 of 17 lungs, the retaining time was designed to be 120 s, but it was variable with 132–425 s in 6 lungs. Time required for other stages varied remarkably as shown in Table 3.

The initial volume of instilled saline ranged within 600–2,300 ml (1,359 ± 435 ml). The initial discharged volume ranged from 150 to 1,282 ml (697 ± 341 ml), with percentage of recovery ranging from 24.3 to 71.4%. The mean volume of instilled saline from the second to the last lavage in each patient varied from 489 to 1,938 ml (893 ± 374 ml). In each cycle, 461–1,896 ml (859 ± 364 ml) of discharged fluid was recovered; the recovery percentage was relatively constant (89.7–100.3%). As a whole, the total volume of saline instilled into each lung was 9,900–28,200 ml, and the total volume of discharged lavage fluid was 8,910–27,000 ml, with total percentage of recovery of 93.4 ± 3.3 (82.1–95.9%).

Simulation of Protein Concentrations in the Drained Lavage Fluid

The theoretical concentrations of IgG, transferrin, albumin, and β_2- microglobulin in the drained lavage fluid were calculated at the end of the draining stage according to the equation described above and by using the procedures detailed in MATERIALS AND METHODS. The theoretical concentrations were plotted on a log scale against time after the beginning of WLL (Fig. 2). The plot for each patient was manually fitted with the protein concentration measured in the drained lavage fluid of each cycle by changing K_s and K_b. Plots for the theoretical concentrations of IgG, transferrin, albumin, and β_2-microglobulin coincide with the measurements (Fig. 2, *A–D*). Data for K_s and K_b are shown in Fig. 3. K_s values for IgG, transferrin, albumin, and β_2-microglobulin ($2.03 \times 10^{-7} \pm 0.902$, $1.95 \times 10^{-7} \pm 0.589$, $1.84 \times 10^{-7} \pm 0.564$, and $1.85 \times 10^{-7} \pm 0.658$, respectively) did not vary among patients (Fig. 3*A*). Importantly, there was no significant difference in K_s values among these four proteins, suggesting that transfer from the surfactant to lavage fluid was independent of molecular weight. However, there was relative variability in K_b among proteins, especially with β_2-microglobulin, which had a K_b that was two orders of magnitude higher than that of the other proteins. K_b values for IgG, transferrin, albumin, and β_2-microglobulin were $4.97 \times 10^{-10} \pm 4.166$, $5.61 \times 10^{-10} \pm 1.990$, $3.82 \times 10^{-10} \pm 1.661$, and $2.28 \times 10^{-8} \pm 0.773$, respectively (Fig. 3*B*). No differences in K_s or K_b values of each protein were found between left and right lungs (data not

Table 2. *Clinical parameters of patients*

	Arterial Blood Gas Analysis			Serum Biomarkers				Pulmonary Function Test			
Case	PaO_2, mmHg	$PaCO_2$, mmHg	A-aDO_2, mmHg	KL-6, IU/ml	SP-D, ng/ml	CEA, ng/ml	GM-Ab, μg/ml	%VC, %	FEV_1/FVC, %	FRC, liters	$\%D_{LCO}$, %
1	81.3	35.8	24.0	7611	963	6.9	6.8	85.8	82.7	2.51	60.5
2	75.6	39.4	25.1	32070	187	12.5	116.0	81.9	79.43	1.77	38.2
3	67.4*	35.5*	269.0*	46700	518	41.4	25.2	51.4	84.5	2.36	19.2
4	55.2*	38.4*	167.7*	26300	799	49.1	25.0	47.7	78.98	1.52	30.0
5	46.4	34.8	60.1	31093	422	19.8	35.7	47.4	98.1	1.41	ND†
6	72.3	38.8	29.2	8356	236	12.8	35.5	85.9	82.9	2.49	70.7
7	51.2	36.7	52.9	10969	407	73.3	6.7	87.5	109.4	1.71	70.8
8	46.2	38.2	55.1	15700	531	16.5	10.4	77.5	80.3	1.53	ND
9	75.3	39.6	24.9	7684	172	7.9	150.9	83.9	75.8	2.50	67.8

Normal Krebs von den Lungen-6 (KL-6), surfactant protein D (SP-D), carcinoembryonic antigen (CEA), and granulocyte/macrophage colony-stimulating factor autoantibody (GM-Ab) levels were within 500 IU/ml, 110 ng/ml, 5.0 ng/ml, 1.0 μg/ml, respectively. *Nasal oxygen supply. †Diffusing capacity of the lung for carbon monoxide (D_{LCO}) of case 5 was not detected because of low vital capacity (VC). ND, not done. A-aDO_2, alveolar-arterial oxygen difference that was measured; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; FRC, functional residual capacity.

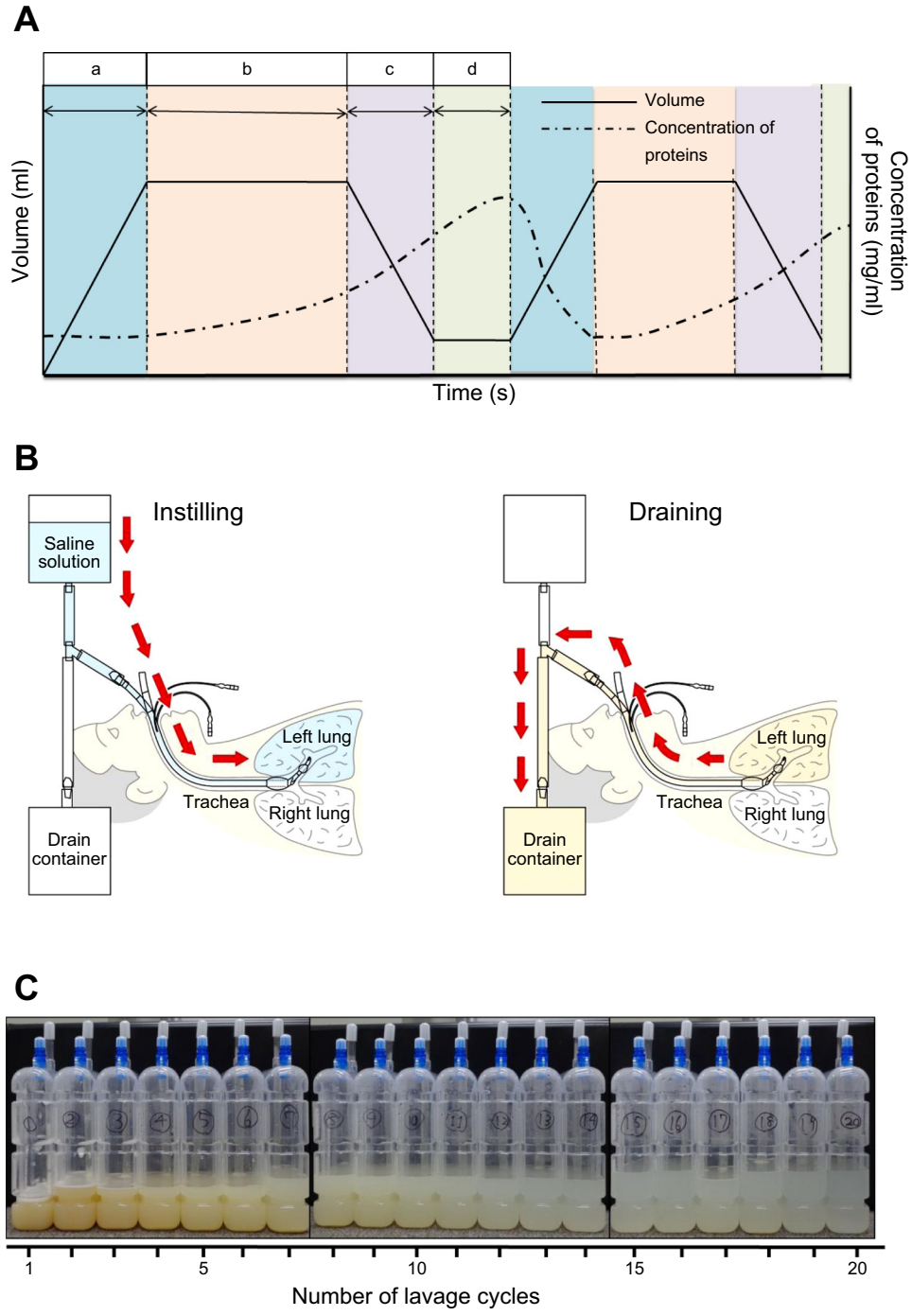

Fig. 1. *A*: conceptual schematic of the time course of the lavage fluid volume in the lung and the concentration of a protein during whole-lung lavage (WLL). Each lavage cycle involved 4 stages: instilling (*a*, from the beginning to the end of saline instillation), retaining (*b*, from the end of saline instillation until the beginning of drainage), draining (*c*, from the beginning to the end of drainage), and preparing (*d*, from the end of drainage to the beginning of the next saline instillation). *B*: schematic of the procedures for instilling (*left*) and draining (*right*) stages. ~0.5 to 2.0 l of saline solution from a bottle was instilled through an endotracheal tube into the lavage lung, retained for a few minutes, and then drained into a draining container. *C*: appearance of the drained fluid obtained from the first to the 20th lavage cycle.

shown). Thus the simulation data confirm the appropriateness of our mathematical model and indicate that the transfer kinetics of proteins into the drained fluid was time dependent.

Durable Effects of the Time on the Lavage Efficiency

To determine the durable effect of each lavage cycle on the slope for the decreasing concentration of each protein in the drained lavage fluid, we evaluated the change in slope of the theoretical curves by varying the duration of the retaining stage in silico. For this purpose, we used the initial data settings in case 4, i.e., the instilling volume of saline; the durations (s) of instillation, retaining, draining, and preparing; and the volume of drained lavage fluid in the first lavage cycle. We found that decreasing curves for the albumin concentration became steeper upon substitution of the shorter time (Fig. 4*A*).

Next, we proceeded to confirm the effects observed in the simulation by using measurements in case 1. We evaluated the rate of declining albumin concentration in the lavage aliquots from a patient who occasionally underwent WLL for the left lung with short-term cycles (120 s, 1–20 cycles) and for the right lung with long-term cycles (540 s, 4–11 cycles). As shown in Fig. 4*B*, the slope of decline for the left lung appeared

Table 3. *Timetable of the stages in each cycle of WLL*

Case	Left/Right	No. of Cycles	Total Time, s	Mean Time ± SD, s*				
				Each Cycle, s†	Stage a, Instilling, s	Stage b, Retaining, s	Stage c, Draining, s	Stage d, Preparing, s
1	L	20	6260	258 ± 15.0	42 ± 3.0	120 ± 1.6	83 ± 42.2	13 ± 18.6
	R‡	11	6895	561 ± 161.7	41 ± 8.1	425 ± 196.2	59 ± 16.4	8 ± 2.6
2	L	20	5200	210 ± 21.9	28 ± 3.0	120 ± 0	52 ± 23.2	13 ± 5.1
	R	20	5450	220 ± 8.3	45 ± 5.9	120 ± 0	52 ± 16.1	8 ± 8.1
3	L	20	5583	237 ± 15.1	36 ± 7.0	120 ± 0	73 ± 18.4	10 ± 2.6
	R	20	6060	236 ± 38.8	35 ± 6.0	120 ± 0	64 ± 26.8	20 ± 16.8
4	L	24	5926	215 ± 19.4	35 ± 4.1	120 ± 0	54 ± 24.7	10 ± 3.3
	R	29	9018	285 ± 17.5	57 ± 3.9	120 ± 0	102 ± 16.8	8 ± 3.3
5	L	20	5230	227 ± 30.6	31 ± 11.9	120 ± 0	76 ± 31.9	11 ± 4.7
	R	20	6480	284 ± 25.3	31 ± 6.2	120 ± 0	121 ± 20.2	19 ± 24.8
6	L	20	5395	266 ± 32.3	10 ± 0	120 ± 0	133 ± 32.0	5 ± 0
	R	20	5680	278 ± 42.2	28 ± 11.2	132 ± 24.6	112 ± 24.8	5 ± 0
7	R	20	6180	283 ± 31.6	52 ± 10.1	120 ± 0	109 ± 41.4	13 ± 11.5
8	L	16	10380	634 ± 264.9	180 ± 32.1	199 ± 28.7	148 ± 50.0	96 ± 250.3
	R	20	11796	550 ± 49.5	188 ± 41.3	200 ± 6.2	153 ± 20.5	16 ± 20.6
9	L	11	6180	553 ± 185.8	146 ± 42.8	193 ± 24.2	174 ± 82.2	40 ± 112.3
	R	13	8280	627 ± 98.1	225 ± 45.2	231 ± 64.1	225 ± 63.3	0 ± 0

*Data are presented as a mean ± SD of time (s) required for 1 lavage cycle. Time for total on each stage of lavage cycle is expressed as a mean ± SD. Instilling time (stage a) is mean time (s) required for instilling saline into the lung. Retaining time (stage b) is mean time (s) applied for retaining saline in the lung. Draining time (stage c) is mean time (s) required for draining lavage fluid to the container. Preparing time (stage d) is mean time (s) required for preparation for the next saline instillation. †Each cycle time is the mean of stage a to d from 2nd to the last lavage. The 1st cycle required 120–1080 s. ‡Time (s) for the 1st 3 cycles ranged within 230–270 s, and that for the 4th to 11th cycles ranged within 625–680 s.

steeper than that for the right lung. The time required to reach 10% of the initial concentration of albumin in the first lavage was 2,730 s for the left lung, whereas it was 4,390 s for the right lung. Notably, both K_S and K_b of the left and right lungs were comparable (1.77×10^{-7} and 4.97×10^{-10} cm/s, respectively, for the left lung; 1.60×10^{-7} and 3.20×10^{-10} cm/s, respectively, for the right lung).

When 1,000 ml of saline was assumed to be instilled into the lung in each cycle, the cumulative amount of albumin drained into the lavage fluid did not differ remarkably within retaining time of 90–570 s (Fig. 4*C*). The curve in short retaining time (90 s) slightly exceeded those in long retaining time (450–570 s) but reversed after 4,000 s. In this setting, the simulation impressed that ~3,200 s (53.3 min) would be required for enough elimination of albumin but that the efficiency of elimination would not significantly change after 5,400 s (90 min).

Effect of Instilled Saline Volume on the Efficiency of WLL

Eqs. 1–3 described in MATERIALS AND METHODS meant that the effect of WLL on elimination of proteins was affected by the instilled saline volume into the lung. As shown in Fig. 4*D*,

Table 4. *Volume balance during WLL*

Case	Left/Right	First Cycle		Second to the Last Cycle			
		Instilled Volume, liters	Drained Volume, liters (recovery %)	Average Instilled Volume, liters	Average Drained Volume, liters (recovery %)	Total Instilled Volume, liters	Total Drained Volume, liters (recovery %)
1	L	1.70	1.05 (61.8)	0.96	0.93 (96.6)	20.0	18.7 (93.7)
	R	1.90	1.10 (57.9)	1.04	1.01 (97.1)	12.3	10.1 (82.1)
2	L	1.40	0.50 (35.7)	0.55	0.51 (91.4)	11.9	10.1 (84.9)
	R	1.50	0.90 (60.0)	0.84	0.81 (96.6)	17.5	16.4 (93.5)
3	L	1.50	1.00 (66.7)	0.85	0.82 (96.3)	16.9	16.6 (93.8)
	R	1.70	0.90 (52.9)	0.88	0.89 (100.3)	18.5	17.8 (95.9)
4	L	0.90	0.37 (41.1)	0.64	0.63 (97.1)	16.5	14.7 (89.3)
	R	1.40	1.00 (71.4)	0.96	0.93 (97.0)	28.2	27.0 (95.7)
5	L	0.60	0.15 (25.0)	0.49	0.46 (94.2)	9.90	8.91 (90.0)
	R	1.00	0.32 (32.0)	0.59	0.56 (95.4)	12.2	11.0 (90.2)
6	L	1.00	0.60 (60.0)	0.55	0.53 (96.6)	11.4	10.7 (93.4)
	R	1.00	0.50 (50.0)	0.63	0.61 (95.8)	13.0	12.0 (92.3)
7	R	1.00	0.50 (50.0)	0.76	0.75 (98.6)	15.5	14.8 (95.5)
8	L	1.10	0.27 (24.3)	0.94	0.85 (89.7)	15.2	13.0 (85.0)
	R	1.30	0.47 (36.0)	0.97	0.95 (98.5)	19.8	18.5 (94.4)
9	L	1.80	0.93 (51.9)	1.58	1.48 (93.8)	17.6	15.7 (89.5)
	R	2.30	1.28 (55.7)	1.94	1.90 (97.8)	25.6	24.0 (94.1)

The instilling volume of the 1st cycle in case 1–7 was determined by the following equations: functional residual capacity (ml) × 0.45 or 0.55 + tidal volume for the left and right lung, respectively. In case 8 and 9, saline was allowed to be instilled into the lung as much as possible from a bottle at 30 cm height from the tracheal tube.

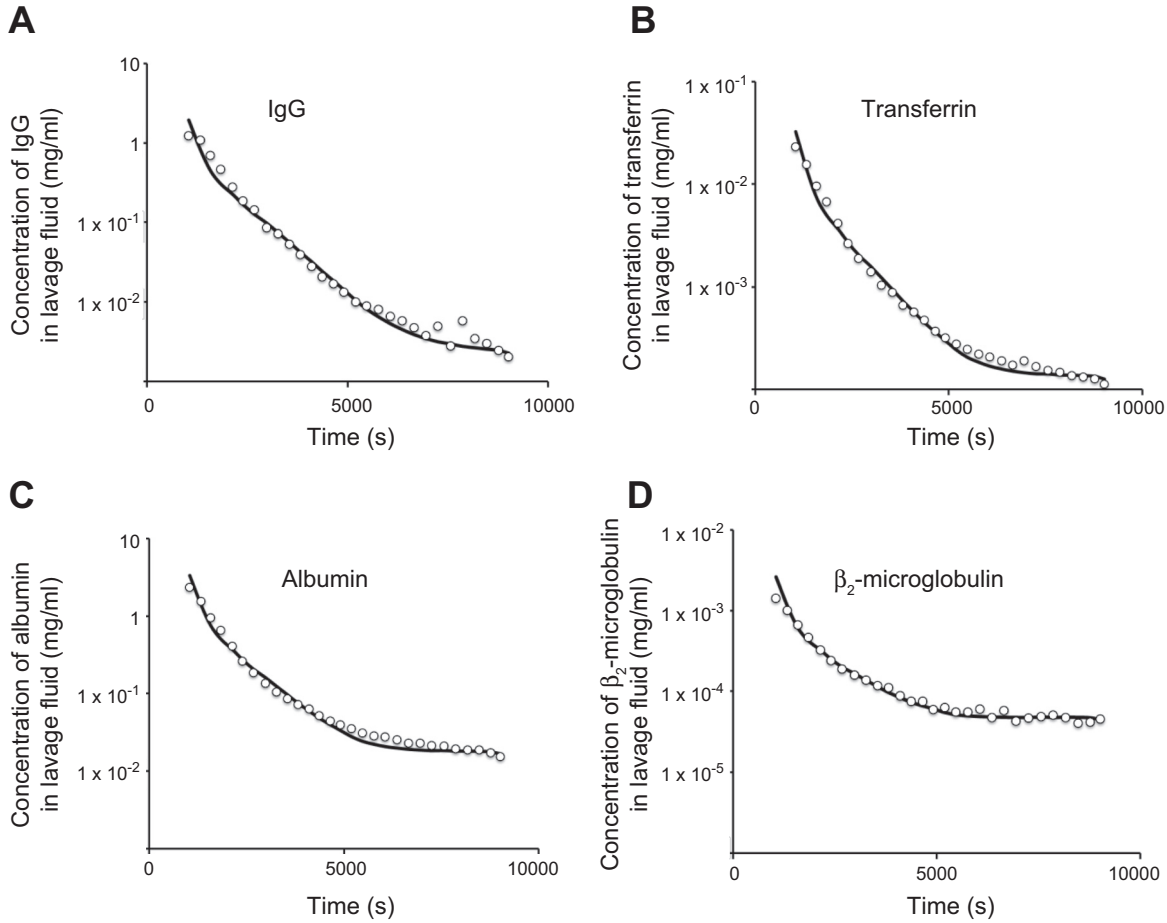

Fig. 2. Theoretical concentrations (lines) and measured concentration (plots) of IgG (*A*), transferrin (*B*), albumin (*C*), and β_2-microglobulin (*D*) in the drained aliquot of lavage fluid for each cycle. The vertical axis is the concentration of the protein on a log scale, and the horizontal axis indicates the time after the beginning of WLL.

when the cumulative eliminated albumin in case 1 was estimated in silico with fixed lavage cycle time at 240 s, the eliminated albumin appeared to increase as the instilled volume increased during 0 to ~3,200 s. After 3,200 s, the eliminated albumin gradually increased, but the volume effect seemed to be diminished.

Exceptional Substances That Fail to Follow the Mathematical Model

Although we applied our mathematical model to the transfer of various substances during WLL, we found that the following substances did not follow the model.

Gastrin and urea. Measured levels of gastrin and urea did not exhibit an exponential decreasing phase but instead reached a plateau in the early stage of WLL (Fig. 5, *A* and *B*). Thus calculation of K_s was difficult. Permeation of gastrin and urea from the blood to the lavage fluid occurred so quickly that the theoretical curves were hardly matched with the actual measurements, which themselves fluctuated markedly during the plateau phase.

SP-D. The SP-D concentration in the drained lavage fluid decreased consistently to a minor extent in the four lungs in the absence of an exponential phase and quickly reached a plateau in the early phase (Fig. 5*C*). As alveolar type II cells and nonciliated Clara cells abundantly release SP-D into the lower respiratory tract, this early plateau phase reflects its active release in situ.

GM-CSF autoantibody. Although the quantified GM-CSF autoantibody belongs to an IgG isotype, theoretical curves of the concentration in the drained lavage fluid did not fit with the measured autoantibody concentration even upon substitution of various sets of coefficients with K_s and K_b in all 17 lungs (Fig. 5*D*).

DISCUSSION

By using a mathematical model based on measured concentrations of proteins, this study investigated the transfer of proteins from the surfactant and blood into the lavage fluid during WLL. We confirmed that the transfer followed a time-dependent differential equation, which assumes that the rate of transfer is proportional to the transmission coefficient, the effective surface area, and the protein gradient between the body compartment and lavage fluid (44).

By using various methods (e.g., comparisons of the protein concentrations between the plasma, sputum, and BALF) and by proving that the IgG1/IgG2 ratio between the BALF and serum are comparable, previous studies demonstrated the transfer of circulating proteins into the alveolar spaces (2, 14, 18, 28, 39).

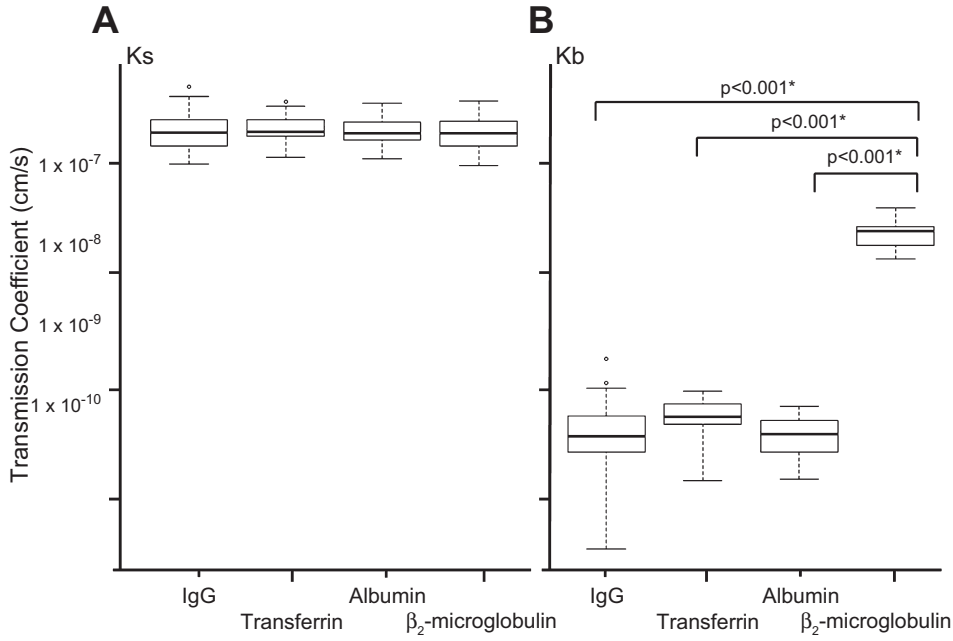

Fig. 3. Coefficients of transfer of IgG, transferrin, albumin, and β_2-microglobulin from surfactant (K_s) (*A*) and blood (K_b) (*B*) to the lavage fluid. The vertical axis indicates the transmission coefficients (cm/s) on a log scale. Statistical significance of coefficients between 2 proteins are shown in the figure.

More recently, intravenously injected GM-CSF autoantibodies were detected in the BALF of nonhuman primates and were observed to reproduce PAP (35). These results indicate that the antibody can cross the air-blood barrier (35). The kinetics of transfer from the blood to the air space and vice versa was studied both in vitro and in vivo (3, 23, 26, 27, 34). In one study, the transmission coefficient (10^{-7}-10^{-5} cm/s) of various proteins across a monolayer of A549 cells was shown to indicate bidirectional transfer. These coefficients appear to be inversely correlated with the molecular weight of proteins (22). In another study, the transmission coefficient for proteins in a monolayer of rat alveolar epithelial cells in vitro was within 10^{-9}-10^{-7} cm/s, whereas that for albumin in sheep lung in vivo was 5×10^{-10} cm/s (11, 17). Thus mass transfer from the blood to the air spaces may be continuously taking place even at steady state.

In previous studies by Ikegami et al. (15), surface tension maintained by surfactant materials covering the alveolar surface was found to have a probable role in interfering with massive transfer and subsequent accumulation of circulating proteins in the air spaces. Interference with the transfer is known to be disrupted by the elimination or deficiency of SP-B (15, 16). Lung lavage may remove surface-active materials in the alveoli and thus temporally disrupt the mechanisms that interfere with the influx of circulating proteins. It is for this reason that we focused on WLL to clarify the mechanism of protein transfer from the blood or surfactant to the lavage fluid. We found that the protein transfer followed a time-dependent mathematical model that was made analogous to the heat transmission model. To our knowledge, this is the first study that has clarified the mechanism of protein transfer in the lung during WLL.

To postulate a mathematical model, we assumed that the transfer of proteins from each body compartment to the lavage fluid consists of two pathways, namely transfer from the accumulated surfactant to the lavage fluid and transfer from the blood to the lavage fluid. The latter may be further divided into two pathways, namely transfer from the blood through the surfactant and direct transfer to the lavage fluid. However, we did not distinguish between these two latter pathways in this study because the transfer of a protein across the air-blood barrier seemed to be rate limiting. We found that protein transfer from the surfactant to the lavage fluid appeared to have K_s values independent of the molecular weight and other properties. It is notable that the K_s values did not differ among patients, indicating the reproducibility of the model. However, mass transfer from the blood to the lavage fluid with variable K_b values did appear to be affected by the molecular weight of the protein because the protein was transferred through a semipermeable membrane consisting of endothelial cells, basement membrane, and type I pneumocytes. Transcytosis was proposed as the primary mechanism of protein transfer for large molecules and of partial paracellular diffusion of small molecules (7, 23). However, the true mechanism remains controversial. As indicated in this study, transfer of β_2-microglobulin (molecular weight of 11 kDa) from the blood to the lavage fluid had K_b values that were two orders of magnitude higher than those of albumin, transferrin, and IgG, which had molecular weights of 66, 80, and 150 kDa, respectively. This difference suggests that β_2-microglobulin diffusion possesses a mechanism that is different from that of other proteins, i.e., it is supposed to be mainly transcytosis for albumin, transferrin, and IgG but mainly paracellular diffusion for β_2-microglobulin. Further analyses will be required to clarify the mechanisms by measuring the permeability of various substances with molecular weight of 10–60 kDa to confirm a "gap" in permeability coefficient K_b among substances with molecular weights in this range.

It is notable that the decrease in concentrations of low-molecular-weight substances in the lavage fluid, namely urea (molecular weight of 60 kDa) and gastrin (molecular weight of 2.1 kDa), was inconsistent with our mathematical model. The measured concentrations appeared to fluctuate and appeared to be independent of time. Moreover, the phase of exponential

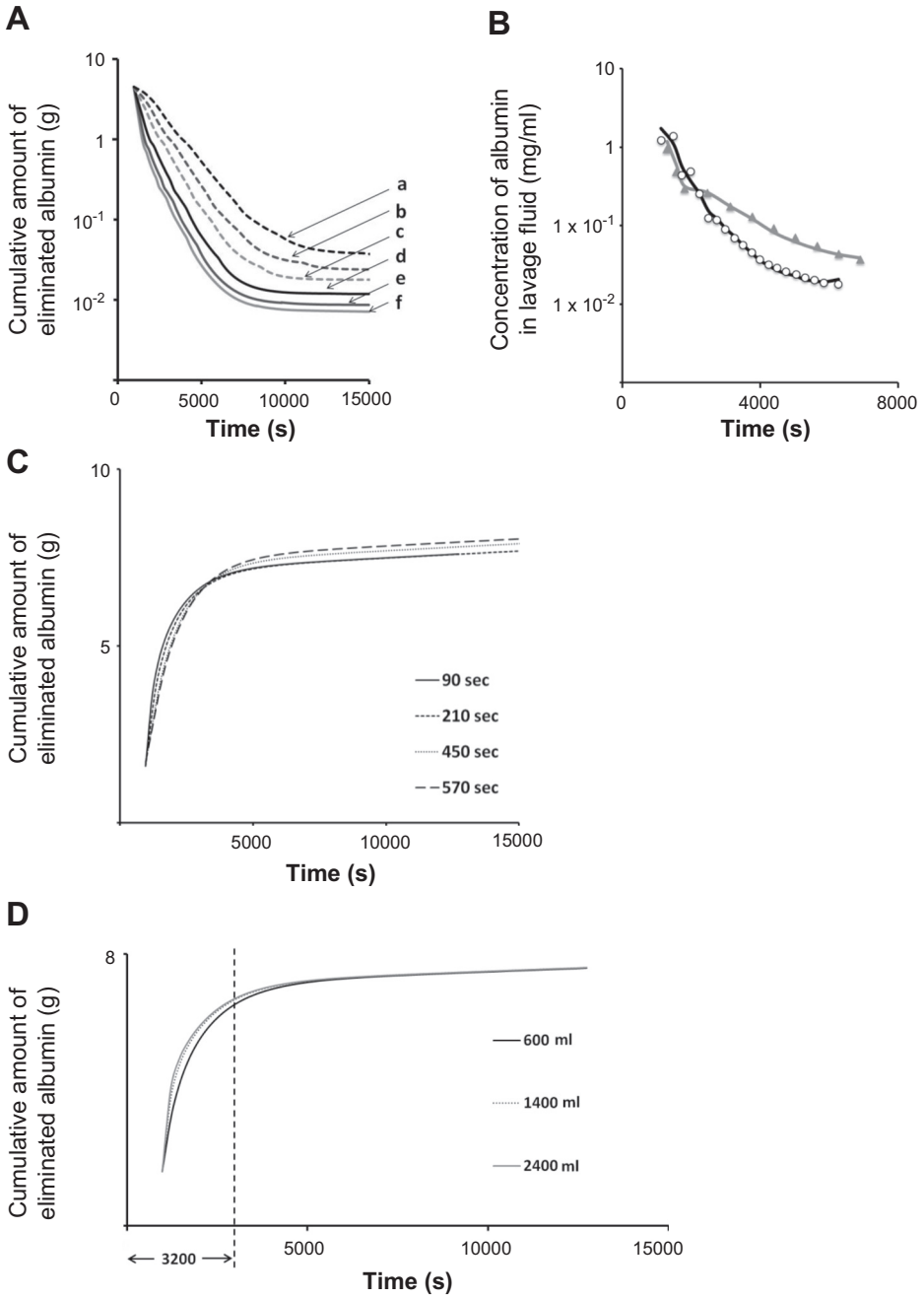

Fig. 4. *A*: durable effect of the retaining stage in each lavage cycle on the theoretical decreasing curve of albumin concentration in the drained lavage fluid. The time assumed for the retaining stage was variable: *a*, 540 s; b, 360 s; *c*, 240 s; *d*, 120 s; *e*, 60 s; and *f*, 30 s. The vertical axis indicates the albumin concentration in the lavage fluid (mg/ml). The horizontal axis indicates the time after the beginning of WLL. *B*: theoretical (lines; black, left; gray, right) and measured (plots; ○, left, ▲, right) concentrations of albumin in the drained lavage fluid in each cycle. The vertical axis indicates the albumin concentration in the lavage fluid (mg/ml). The horizontal axis indicates the time after the beginning of WLL. *C*: simulation curves of cumulative amount of albumin drained in the drained lavage fluid when the retaining time varied with 90 (solid line), 210(small dashed line), 450(dotted line), or 570(large dashed line) s. *D*: cumulative amount of eliminated albumin in the drained lavage fluid. An in silico evaluation by changing instilled saline volume varied with 600 (black solid line), 1,400 (dotted line), or 2,400 (gray solid line) ml.

decrease was hardly defined in six out of ten lungs examined; when there was any decrease, the phase lasted within 1,000 s after the start of WLL (data not shown). This characteristic was likely due to the high permeability of the air-blood barrier to the molecules. Similarly, Rennard et al. (32) reported that urea was more able than glucose and albumin to permeate into the lavage fluid, as observed in normal volunteers with saline instilled into their lung segments.

SP-D is produced by alveolar type II cells and nonciliated Clara cells in the lower respiratory tracts and is secreted into the air space (43). Although SP-D is detectable in the sera of patients with aPAP, its levels are much lower than those of BAL (12). Thus SP-D transfer from the blood to the air space is negligible. The high concentration of SP-D in the lavage fluid was likely due to its continuous production in the lung. The rate of its production was estimated to be 6–13 mg/h on the basis of evaluation of four lungs (data not shown).

The lung is the organ that most abundantly produces GM-CSF, a factor that is critical for terminal differentiation of alveolar macrophages, as it promotes the expression of the transcription factor, PU.1 (38). It is suggested that IgG-type GM-CSF autoantibody is pathogenic and is known to be transferred from the lung capillaries into the air spaces immediately formed by GM-CSF autoantibody complex to become undetectable by our GM-CSF autoantibody ELISA system (30).

Furthermore, we had better to reconsider the adequacy of the present mathematical model when it was applied to substances

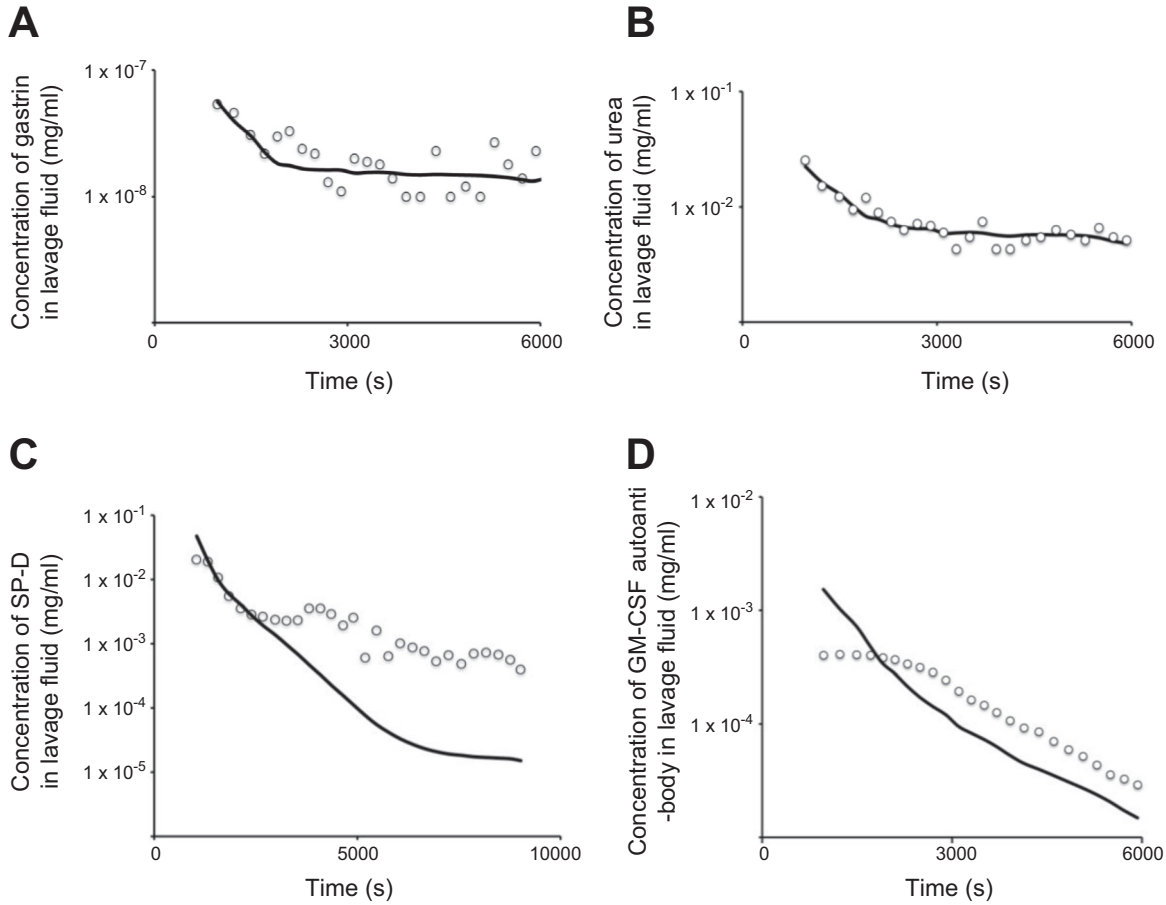

Fig. 5. *A* and *B*: actual measurements (plots) of gastrin or urea concentration in the drained lavage fluid did not exhibit the exponential decreasing phase but reached a plateau fluctuating in the early term. These seemed to migrate immediately from the blood to the lavage fluid. Thus the theoretical curves (lines) were hardly fitted with the actual measured concentration. *C*: concentration of surfactant protein D (SP-D) in the drained lavage fluid revealed slight decrease without exponential phase and soon reached a plateau phase in the early term. As SP-D is abundantly released from alveolar type II cells into the lower respiratory tracts, this early plateau phase probably reflected the active release in situ. *D*: actually measured granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibody concentrations were consistently under the theoretical curve especially in the early stage.

with lower molecular weights by assuming two permeation coefficients, such as K_{b1} (coefficients from the blood to the lavage fluid through surfactant) and K_{b2} (from the blood directly to the lavage fluid).

In the present study, the recovery rate in the first draining lavage fluid was lower than those after the second lavage. Although the first instilled saline remained in the lower respiratory tracts, we did not mind the remaining volume at the first draining because we thought that the remaining lavage fluid could be recovered after the second draining. Therefore, we did not intentionally extend the first draining time longer than those of other cycles. Although we usually perform percussion or vibration on the patient's chest, the recovery rate at the first draining was not improved by these procedures. It is likely that the low recovery rate and its variability of the first lavage shown in Table 4 were due to the early cessation of the first draining.

To date, methods of WLL for the treatment of PAP have not been standardized (25). Michaud et al. (29) recommended instilling 1 l of saline into the lavage lung and then to clamp the draining tube for 4–5 min (29). Bonella et al. (4) and Paschen et al. (31) determined the number of lavage cycles by measuring the optical density of each lavage fluid. They applied statistical evaluation to data from a number of WLLs to find the relationship between instilled saline volume and eliminated proteins. Although their approach is fundamentally different from ours, their finding that instilling volume is an important element for determining the amount of eliminated protein was confirmed in this study (Fig. 4*D*). The protocol for WLL used in this study were variable among participating hospitals, and thus time of each cycle varied between 213–630 s, including 120–540 s for the retaining time. As for our mathematical model, the number of cycles and the retaining times did not influence the efficiency of WLL. Based on *Eq. 1*, the amount of proteins eliminated by WLL was dependent on time after the beginning. According to the volume effect demonstrated by in silico simulation in this study (Fig. 4*D*), larger instilled volume appeared to improve the efficiency of lavage. However, the simulation also suggested that the effect is limited within some range of time. Previous studies, however, demonstrated the volume effect (4). In this regard, total eliminated albumin concentration significantly correlated with instilling saline volume in actually measured values in 17 WLLs of the present study with Rho value at 0.69. However, we have to consider the possibility that it also prolonged the duration of instilling and draining time, and thus longer time for each lavage cycle increases the elimi-

nated protein(s). Thus our mathematical model may be useful to predict the amount of eliminated proteins at a certain time point after the beginning of WLL.

In conclusion, we demonstrated that protein transfer in the lung during WLL followed a relatively simple, mathematical model based on diffusion and that this model could be expressed in terms of a number of differential equations. As an exception of the present mathematical model, substances with low molecular weight do not follow the theory. Our study, not only contributes to the design of an efficient regimen for WLL, but also reveals the mechanism of delivery of specific large drug molecules across the air-blood barrier, such as antibody drugs.

APPENDIX

The Effective Alveolar Surface Area

The effective alveolar surface area was calculated from the data for the alveolar volume, V_A according to the following equations: $A_s = 6.4 \cdot 10^3 \cdot V_A^{2/3}$. For a person with 74 kg body wt, both A_s and V_A were reported to be 143 m^2 and 3,338 ml, respectively (10). The effective surface area of the pulmonary capillaries, A_b, was estimated from the following formula (10): $A_b = 0.89 \cdot A_s$. The relationship between alveolar surface area, S_A, and alveolar volume, V_A, depends on the number of alveoli. S_A increases as the number of alveoli increases at a fixed value of V_A. According to Ref. 10, the average lung volume is 4,300 ml, and the average alveolar surface is $(143 \pm 12) \times 10^4$ cm^2 in normal subjects with an average body weight of 74 kg at 19–40 yr of age. Under these conditions, air-space volume density is 0.865 ± 0.013 cm^2/cm^3, and alveolar surface density is 370.6 ± 28.9 cm^2/cm^3.

We set

$$\beta = \frac{S_A^{\frac{1}{2}}}{V_A^{\frac{1}{3}}} \qquad (A1)$$

where, the right side of the equation is an expression for the constant shape parameter, β.

According to the report described above (V_A and S_A in the space V)

$$\frac{S_A}{V} = 370.6 \text{ cm}^2/\text{cm}^3 \qquad (A2)$$

$$S_A = 143 \times 10^4 \text{ cm}^2 \qquad (A3)$$

$$\frac{V_A}{V} = 0.865 \text{ cm}^3/\text{cm}^3 \qquad (A4)$$

From *Eqs. A2* and *A3*,

$$V = 3859 \text{ ml} \qquad (A5)$$

and from *Eqs. A4* and *A5*

$$V_A = 3338 \text{ ml} \qquad (A6)$$

where the anatomical dead space is 4,300 − 3,338 = 962 ml. Introducing *Eqs. A3* and *A6* into *Eq. A1*,

$$\beta = \frac{\sqrt{143 \times 10^4}}{\sqrt[3]{3338}} = 80.02 \qquad (A7)$$

On the basis of *Eq. A1* (note that S_A is in m^2 and V_A is in ml),

$$S_A = \beta^2 \cdot V_A^{\frac{2}{3}} = 6.403 \times 10^3 \cdot V_A^{\frac{2}{3}} \qquad (A8)$$

The value of β may be considered as constant even with a change in V_A in the same subject, as the number of alveoli and the shape do not change, particularly in the supine position.

Method for Optimizing the Transmission Coefficients

A program was written in Visual Basic Application using various coefficients to calculate the theoretical substance concentrations in the lavage aliquots. For explanation, we show an example of simulation used to obtain the best fitting curve shown in Fig. 2*C*. As shown in Appendix Fig. A1*A*, the value for K_s could be determined to be 1.8×10^{-7} cm/s by the least-square method until 3,000 s when K_b was assumed to be 0 cm/s. Next, K_b value was determined to be 5.2×10^{-10} cm/s again by the least-square method by 9,018 s. As shown in Appendix Fig. A1*B*, the theoretical curve appeared closer to the dotted actual measurements. Then K_b was changed to 6.1×10^{-10} cm/s manually, as shown in Appendix Fig. A1*C*; the theoretical curve

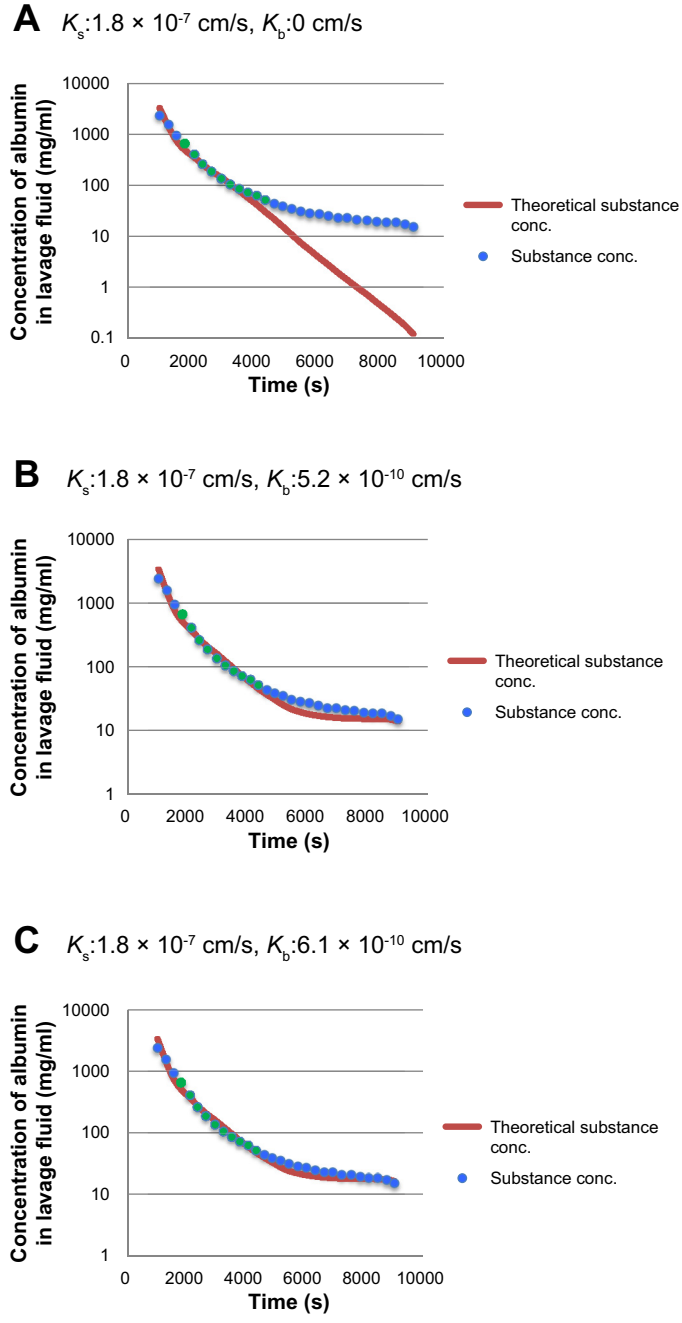

Fig. A1. Example of simulation used to obtain the best fitting curve shown in Fig. 2*C*.

completely coincides with the dotted actual measurements. Therefore, K_b was determined to be 6.1×10^{-10} cm/s.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.A., T.M., K.U., R.T., T.I., Y. Inoue, and K.N. conception and design of research; K.A., A.H., Y. Ito, H.W., T.W., Takero Arai, H.M., S.O., R.T., T. Takada, E.Y., T.I., M. Hirose, and Toru Arai performed experiments; K.A., T. Tanaka, T.M., N.K., M. Hayashi, M. Hirose, and K.N. analyzed data; K.A., T. Tanaka, T.M., N.K., R.T., E.Y., H.K., and K.N. interpreted results of experiments; K.A., T. Tanaka, R.T., and K.N. prepared figures; K.A., T. Tanaka, T.M., K.U., T.I., and K.N. drafted manuscript; K.A., T.M., K.U., R.T., Toru Arai, H.K., and K.N. edited and revised manuscript; K.A., T. Tanaka, T.M., N.K., A.H., H.W., Takero Arai, M. Hayashi, H.M., K.U., S.O., R.T., T. Takada, E.Y., T.I., M. Hirose, Toru Arai, Y. Inoue, H.K., and K.N. approved final version of manuscript.

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