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An Immunocytochemical Study on Human Pulmonary Surfactant and Its Application to Legal Medicine

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ABSTRACT **Pulmonary surfactant is believed to contribute to decrease the alveolar surface tension to maintain the alveolar spaces. Monoclonal antibodies against human pulmonary surfactant apoproteins of 34-37 kDa were prepared. Indirect immunocytochemistry using this antibody was performed on the paraffin sections of pulmonary tissue obtained from newborns, stillborns and infants at autopsy. Results showed the staining profiles of newborn and stillborn specimens to be slightly different from those of infants, and also to be altered according to the degree of alveolar expansion or degeneration and/or putrefaction. The author confirmed the existence of apoproteins even in the putrefied pulmonary tissue of a case examined at 3 or 4 months after death. This staining method clarified that a newborn had died of Respiratory Distress Syndrome, ruling out the first suspected cause of infanticide. This paper discussed the usefulness of the antibody and its application to legal medicine.**

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Key Words: Pulmonary surfactant, Surfactant apoprotein, Monoclonal antibody, Immunoperoxidase staining, Respiratory Distress Syndrome (RDS).

1 Introduction

Pulmonary surfactant, which covers the surface of the alveoli, is believed to prevent alveolar collapse by lowering surface tension[1]. This substance is believed to play an important role for the alveolar expansion after the first air breathing at birth, and for maintaining the small air spaces of alveoli open afterwards[2,3].

Human pulmonary surfactant is a complex which contains about 90 percent lipids and about 10 percent proteins[3]. The main components of lipids are phospholipids, of which the principal ingredients are dipalmitoyl-phosphatidylcholine and phosphatidyl glycerol[3]. Human surfactant apoproteins are composed of 36 kDa and 62 kDa protein groups and the former is also called 34-37 kDa protein group[5]. The proportion of phospholipids and apoproteins is generally very similar in mammalian surfactants regardless of their species. Moreover, the molecular weight of each apoprotein is very similar among animal species. Previous studies using polyclonal antibodies against the apoproteins of rat[12-14] and rabbit[11] have been performed on pulmonary tissues in each stage of development, and the localization of the apoproteins in the pulmonary tissues has been demonstrated.

Recently, monoclonal mouse antibodies against human pulmonary surfactant apoproteins were prepared by Kuroki *et al.*[5], and it has become possible to recognize the existence of human pulmonary surfactant by light-microscopy with higher sensitivity using the immunoperoxidase staining method.

In this paper, we reported the usefulness of the antibodies in its application to legal medicine. We also reported an interesting case, in which the antibody was effective to certify the cause of death of a newborn as Respiratory Distress Syndrome (RDS).

2 Materials and Method

2·1 Materials: The pulmonary tissues of 25 newborns and 6 stillborns, and 8 infants were used. With the exception of case No. 31, they were all collected by legal autopsies performed in our department from June, 1981 to April, 1986. Samples were taken from the right upper pulmonary lobes.

2·2 Preparation of thin sections: The lungs were fixed with 20% formalin or 20% phosphate buffered saline (PBS) formalin and embedded in paraffin. Thin sections of about 3μm in thickness were dried on glass slides coated with egg albumin. Deparaffinization with xylene and alcohol series was followed by incubation in Kardasewitsch solution (5% ammonia solution 5ml+70% ethanol 100ml) for half an hour to eliminate formalin pigments.

2·3 Immunoperoxidase staining: The sections were stained by the avidin-biotin peroxidase complex (ABC) method, which is thought to have the highest sensitivity of all the peroxidase stainings[15]. There are two types of monoclonal antibodies named PC6 and PE10[5], and we used PE10 as the first antibody in the present study. The prepared sections were incubated in 0.3% H_2O_2 in methanol for the inhibition of endogenous peroxidase for 30 minutes, and then incubated in 10% normal serum for 10 minutes to prevent the background staining. Next, the sections were incubated with the monoclonal antibody for 30 minutes, and then with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, Calif.) for 30 minutes. The sections were incubated further with avidin D-biotinylated horseradish peroxidase complex (Vector Laboratories, Inc., Burlingame, Calif.) solution for 30 minutes. Peroxidase activity was revealed by incubating the sections in 0.05 M 3,3'-diaminobenzidine-tetrahydrochloride (Katayama Chemicals Co., Osaka, Japan) in 0.1 M tris-HCl, pH 7.6, containing 0.01% H_2O_2. Finally, the sections were counter-stained with hematoxylin for 5 seconds. All the incubations were followed by three rinses in PBS-solution.

2·4 Other stainings: To evaluate the postmortem changes of the tissues, the sections which were adjacent to those stained by immunoperoxidase staining were stained by hematoxylin-eosin in all cases (H-E sections). Another immunoperoxidase staining using anti-keratin antibodies (DAKO Corporation., Santabarbara, U. S. A.) as the first antibody was also performed to compare the distribution of kematin fibers and that of surfactant.

2·5 Evaluation: The following criteria were adopted to judge whether the specific reaction was positive or not. When reaction products, which were considered to be the products of antibody-antigen interactions, were observed in the specimens, judged a positive reaction whether the reaction was seen in the alveolar spaces or within the alveolar type II cells. We differentiated the non-specific and specific reactions from the results of negative control studies in which the first antibody was excluded. We also judged a positive result when positive reaction products were distributed on the specimens, even if the cells or tissue organizations had already disintegrated due to the putrefaction.

3 Results

Table 1 shows the summary of the cases. The results of the immunoperoxidase staining for each case are summarized in Table 2, and other morphological findings are shown in Table 3. The results of immunoperoxidase staining for respective cause of death, listed from the earliest to the latest postmortem lapse of time, are shown in Table 4 and Table 5.

3·1 Evaluation of the postmortem changes;

3·1·1 Morphological changes of lungs: By light-microscopy, pulmonary tissues generally demonstrated the following changes. Up to 3 days after death, the tissues did not show any particular changes. Exfoliation of the epithelial cells of bronchioli was often seen in the specimens of 3 or 4 days after death. In the tissues of approximately one week after death, the contours of the cells gradually became vague and

Table 1-1 *Summary of the cases (newborn and stillborn) : The cases were listed from the earliest to the latest postmortem lapse of time.*

Case No.	Cause of death	Items	Period of gestation	Weight (g)	Lapse of time	Time of autopsy
1	Asphyxia	drowning*2	term	3,068	12-36 hr.	Jan.
2	Asphyxia	nas. ob.*3	term	2,770	18-30 hr.	Apr.
3	Injuries	tr. ac.*4	term	2,450	20-28 hr.	Aug.
4	Disease	RDS	34 wk.	2,750	20-30 hr.	Mar.
5	Asphyxia	drowning	term	3,380	24 hr.	Jul.
6	Asphyxia	drowning	term	2,510	24-26 hr.	Apr.
7	Stillborn		30-35 wk.	1,940	24-28 hr.	Apr.
8	Asphyxia	drowning	term	2,700	24-36 hr.	Jul.
9	Asphyxia	drowning	term	3,000	24-36 hr.	Oct.
10	Asphyxia	strang.*5	term	3,160	36-48 hr.	Nov.
11	Asphyxia	drowning	term	2,620	40- 4 hr.	Sep.
12	Asphyxia	strang.	term	3,070	48-60 hr.	Mar.
13	Stillborn		term	2,770	2 da.	Apr.
14	Asphyxia	drowning	term	3,000	2- 3 da.	May.
15	Asphyxia	unknown	term	3,850	2- 3 da.	Dec.
16	Asphyxia	unknown	term	2,480	2- 3 da.	Jan.
17	Cold*1	left outdoors	term	2,600	2- 3 da.	Jan.
18	Asphyxia	unknown	32-36 wk.	2,330	3- 4 da.	Nov.
19	Asphyxia	unknown	term	3,000	3- 4 da.	May.
20	Asphyxia	strang.	term	2,250	3- 7 da.	Nov.
21	Asphyxia	unknown	36 wk.	2,530	4- 7 da.	Jun.
22	Asphyxia	nas. ob.	term	2,800	7-10 da.	Jun.
23	Asphyxia	strang.	term	2,850	7-10 da.	Jan.
24	Asphyxia	strang.	term	2,430	7-14 da.	Dec.
25	Stillborn		term	2,100	10-30 da.	Jul.
26	Stillborn		term	2,865	1 m.	Aug.
27	Asphyxia	resp. fail.*6	term	2,600	1- 2 m.	Jun.
28	Stillborn		28-32 wk.	1,880	1- 6 m.	Apr.
29	Asphyxia	unknown	term	2,800	3- 4 m.	Nov.
30	Asphyxia	drowning	term	2,200	3- 4 m.	Mar.
31	Stillborn	miscarriage	20-22 wk.	360	1- 2 da.	Sep.

Table 1-2 *Summary of the cases (infants).*

Case No.	Cause of death	Items	Age	Lapse of time	Time of autopsy
1	Disease	SIDS*7	8 m.	10-12 hr.	Jun.
2	Disease	ac. br. pn.*8	1 yr.	12-15 hr.	Jul.
3	Disease	SIDS	52 da.	12-24 hr.	Jan.
4	Disease	SIDS	4 m.	15-20 hr.	Dec.
5	Disease	SIDS	8 m.	24 hr.	Jul.
6	Asphyxia	strang.	16 m.	24-30 hr.	Jun.
7	Asphyxia	strang.	5 yr.	2- 3 da.	Jan.
8	Asphyxia	strang.	1 yr.	6 m.	Feb.

*1 : Death from cold.
*2 : Death by drowning ; all cases were found in toilet tanks.
*3 : Naso-oral ogstruction.
*4 : Traffic accident ; corpse was found on the street.
*5 : Strangulation.
*6 : Respiratory failure.
*7 : Sudden Infant Death Syndrome.
*8 : Acute broncho-pneumonia.

Table 2-1 *Results of immunoperoxidase staining of newborn and stillborn specimens.*

Case No.	Surfactant		Type II cells*3	
	Amount*1	Distribution*2	Number	Intensity of reaction
1	1+~2+	lu・II・wa	++	weak
2	1+~2+	lu・II・wa	++	weak
3	2+	lu・II	++	
4	−	−	※	
5	2+	lu・II	++	
6	3+	lu・II	++	
7	2+	u・II	++	
8	2+	II	++	
9	2+	lu・II	++	
10	2+	II	+(++?)	weak
11	2+	lu・II	++	weak
12	2+	lu	※	weak
13	2+~3+	II	++	
14	2+	lu・II	++	
15	2+	lu	※(++?)	
16	2+	lu・II	※	
17	2+	lu・II	++	
18	3+	lu・wa	※	
19	3+	lu・II	++	weak
20	2+	lu・II	++	weak
21	3+	lu・II・inf	++	
22	3+	lu・II (?)	+(++?)	
23	3+	lu・II・wa	++	
24	3+	lu・II・wa	++	
25	2+	inf.	−	
26	1+	inf.	−	
27	2+	inf.	−	
28	1+	inf.	−	
29	−	−	−	
30	3+	lu・II (?)	※	
31	1+	lu・II (?)	++	

Table 2-2 *Results of immunoperoxidase staining of infants.*

Case No.	Surfactant		Type II cells	
	Amount*1	Distribution*2	Number	Intensity of reaction
1	2+	II・wa	++	
2	2+	lu・wa	※	weak
3	2+	lu・II・wa	+	weak
4	2+	II・wa	++	
5	2+	II	+	weak
6	2+	lu・II・wa	++	weak
7	2+	lu・II・wa	++	weak
8	−	−	−	

*1 amount ; 1+ : little, 2+ : normal, 3+ : much
*2 distribution ;
lu : in alveolar lumen
II : inside alveolar type II cells
wa : adhering to alveolar wall
in. : infiltrated and/or adsorbed by degeneration products
*3 type II cells ; ++ : numerous
+ : relatively few
※ : difficult to differentiate
− : none observed

Table 3-1 *Other findings (newborn and stillborn).*

Case No.	Alveoli Exp.*1/Unexp.*2	Other profiles
1	G /almost none	
2	NG/partly	alveolar collapse
3	G /scattered	hemorrhage in bronchioli
4	− /most	a few dilated alveoli and bronchioli
5	NG/most	bronchioli slightly dilated
6	NG/much	odd things in bronchioli
7	− /most	bronchioli slightly dilated
8	G /partly	
9	G /partly	odd things in bronchioli
10	G /almost none	
11	G /partly	
12	G /scattered	
13	− /most	alveolar edema / denucleated type II cells
14	G /partly	odd things in bronchioli
15	G /partly	type II cells released off surfactant(?)
16	− /most	bronchioli partly dilated
17	G /scattered	broncho-epithelium exfoliated
18	NG/much	
19	G /scattered	broncho-epithelium exfoliated
20	G /almost none	
21	G /much	broncho-epithelium exfoliated
22	NG/much	abundant type II cells denucleated(?)
23	G /partly	
24	G /partly	
25	− /most	putrefaction(+) / nucleoli(+)
26	? / ?	putrefaction(+++) / cell(−)
27	NG/most	putrefaction(++) / nucleoli(+)
28	? / ?	putrefaction(++) / cell(−)
29	? / ?	putrefaction(++) / cell(−)
30	NG/much	putrefaction(+) / denucleated type II cells
31	− /	

Table 3-2 *Other findings (infants).*

Case No.	Alveoli Exp.*1/Col.*3	Other profiles
1	G / (−)	alveolar edema(+)
2	G / (−)	alveolar edema/hemorrhage/inflammation
3	G / (−)	alveolar edema(++)
4	G / (−)	alveolar edema(+)
5	G / (−)	thickened septa / congestion
6	G / (−)	alveolar edema(++)
7	G / (−)	alveolar edema(++)
8	? / ?	putrefaction(+++)/cell(−)

*1: expansion of alveoli;
G: good, NG: not good,
−: not expanded, ?: unable to judge
*2: distribution of unexpanded area
*3: alveolar collapse

Table 4-1 *Summary of causes of death and results of immunoperoxidase staining (newborn and stillborn).*

	Cause of death		Items	Positive	Negative
1	Asphyxia	22	drowning	8	0
			strangulation	5	0
			nas. obst.*2	2	0
			unknown	6	1
2	Disease	1	RDS*3	0	1
3	Injuries	1	tr. accident*4	1	0
4	Cold*1	1	left outdoors	1	0
5	Stillborn	5	SFD*5	3	0
			AFD*6	2	0
	Total	30		28	2

Table 4-2 *Summary of causes of death and results of immunoperoxidase staining (infant).*

	Cause of death		Items	Positive	Negative
1	Asphyxia	3	strangulation	2	1
2	Disease	5	SIDS*7	4	0
			ac. br. pneum.*8	1	0
	Total	8		7	1

*1: Death from cold
*2: Naso-oral obstruction
*3: Respiratory distress syndrome
*4: Traffic accident
*5: Small-for-date
*6: Appropriate-for-date
*7: Sudden Infant Death Syndrome
*8: Acute broncho-pneumonia

denucleation of the alveolar type II cells was remarkable. In the tissues which were estimated to be more than a month old, there were so much putrefaction that individual cells were not discernible. In general, the lower the temperature of the environment in which the corpse were kept, the less degeneration of tissues occurred.

Table 5 *Results of immunoperoxidase staining summarized according to the postmortem lapse of time (newborn and stillborn).*

Lapse of time		Result		Remarks
≦1 wk.	21	positive	20	
		negative	1	RDS
1 wk.~1 m.	4	positive	4	
		negative	0	
1 m. ≦	5	positive	4	
		negative	1	putrefied

3·1·2 Changes in the immunoreactive products: Reaction products were discernible in the pulmonary tissue as late as 3 and/or 4 months after death, the longest period of all. The localization and appearance of the reaction products observed were different according to the degree of alveolar expansion or the lapse of time after death.

i) The early stage (within 2 or 3 days after death): At this stage the reaction products were localized

in alveoli, bronchioli, and within the cytoplasm of the alveolar type II cells. The alveolar epithelial cells (type I cells), endothelial cells and other interstitial cells did not showed any similar reactions. The same results were seen in both newborn and stillborn cases (Fig. 1).

When the inhibitory effect on endogenous peroxidase was insufficient, the granulocytes and monocytes showed a similar positive reactions with light-brown staining. In the infants, the macrophages, which appeared scarcely in newborn tissues, ingested a large amount of surfactant and showed a similar reaction in their cytoplasm. They sometimes resembled alveolar type II cells, but they were easily differentiated from the cells by their shape and localization.

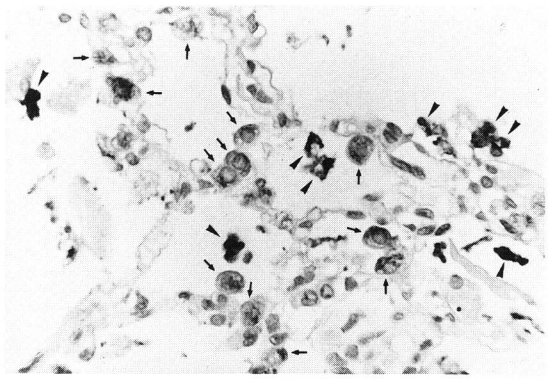

Fig. 1 (Case No. 8) Type II cells (arrows) and amorphous granular masses (arrow heads) showed the specific reaction. No positive reaction was demonstrated in alveolar epithelial cells, vessels or other interstitial cells. The type II cells sometimes resembled alveolar macrophages. (400×)

ii) From 1 to 2 weeks after death: The surface of alveoli was often coated linearly with light-brown colored reaction products that were occasionally stained darkly. It became more difficult to recognize the alveolar type II cells because of the thinner density of the specific reaction in the cytoplasm and the deformity of cells due to degeneration.

iii) From 2 to 4 weeks after death: Degeneration was highly advanced, and most of the cells had collapsed and deposited degeneration products at their original sites. Specific reactions were observed in the degenerative products, and they appeared as if they surrounded the alveolar lumina. It was impossible to recognize the alveolar type II cells.

iv) More than a month after death: Almost no cells were observed in the specimens because of highly advanced degeneration and/or putrefaction. However even in such specimens, reaction products sometimes were discernible among the degenerative products, although they were scarce in amount (Fig. 2).

3·2 Newborn and stillborn cases: Examination of the lungs of stillborns and newborns who died immediately after birth without sufficient respiration revealed the specific reactions mainly in the alveolar lumina, which had still not expanded and contained no air spaces. The alveolar type II cells also showed a positive reaction. Under low magnifying power, numerous large masses of reaction products which filled up almost all the alveolar lumina were observed (Fig. 3). Higher magnifying power revealed innumerable fine reaction products that adhered to the shed epithelial cells or to the keratin fibers, surrounding them like a core. These masses of fine granules were believed to be surfactant, which were stored for the preparation of alveolar expansion after birth[4].

In the cases of newborns whose alveoli were sufficiently expanded with air, the reaction products were generally observed only in the type II cells (Fig. 1). In the alveolar lumina, some reaction products were seen coating the surface of the alveoli linearly with extremely thin density. Occasionally, a few

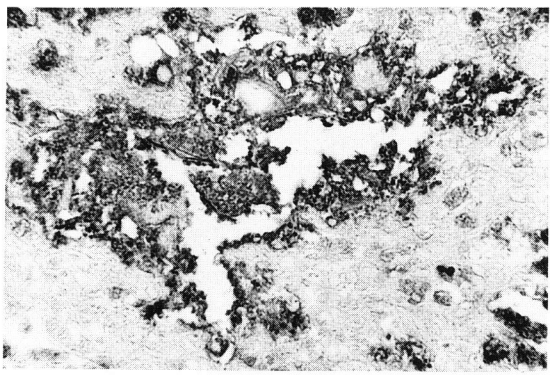

Fig. 2 (Case No. 27) No nucleoli or alveolar type II cells were recognizable in this specimen. Specific reactions were observed in the degenerative products, which appeared to surround the alveolar lumen. (400×)

small granular reaction products adhering to the surface of the alveoli and bronchioli were recognized, and they were speculated to be the remainder of stored surfactant.

3·3 Infant cases: The H-E sections of almost all the cases showed pulmonary edema and pulmonary congestion. The staining profiles of the reaction products were basically similar to those of the newborns, with the exception of their distribution, which was slightly different. The positive reaction observed in the alveolar type II cells was similar to that observed in newborn type II cells. The large masses of reaction products that were often observed in the newborn specimens were not observed in the infant specimens, but small amounts of reaction products were seen attached to the surface of alveoli.

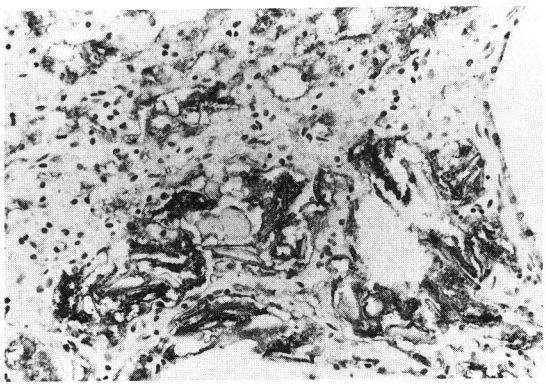

Fig. 3 (Case No. 6) Alveoli, which are not expanded yet, reveal large masses of reaction products. Most of the fine granules of the reaction products seemed to adhere not to the alveolar surface but to keratin fibers. (200×)

3·4 A legal autopsy case: The following is a summary of an interesting case in which an elderly midwife was accused of accidental infanticide, which was ruled out after investigation because the results of the immunoperoxidase technique confirmed the cause of death to be Respiratory Distress Syndrome.

3·4·1 Summary of the case: The mother of the newborn was a 33-year-old housewife who was in her second pregnancy.

On the early morning of the 15th of March, 1985, The baby, a male, was delivered after 34 weeks of gestation. He was 46.5 cm in height, and 2,750 g in weight. Witnesses at the birth testified that the baby had cried after delivery. At 8: 00 a. m., after all routine procedures had been completed, the midwife returned home, having noticed nothing amiss with the mother and baby. The husband's sister, who lived next door to the newborn's family, checked on the baby frequently after the delivery while the mother slept. At about 12: 30 p. m., the baby was found dead.

On March 16, a legal autopsy was performed on suspicion of accidental infanticide and/or inadequate care of the baby, about 20 hours after the death.

3·4·2 Autopsy findings: There were no remarkable changes in the external appearance of the infant. But there were many petechiae under the pulmonary pleura and the pericardium. Results of the hydrostatic tests revealed that the stomach and intestines were floating on the water but that the lungs were sunk to the bottom, though it had been confirmed that the baby had breathed at birth.

Microscopic observation of the sections of the lungs revealed that only a few alveoli and bronchioli were dilated with air. Most of the alveolar lumina was occupied by eosinophilic fluid. Immunoperoxidase staining revealed that the small air spaces of alveoli could not remain open because pulmonary surfactant was completely absent at the time of death (Fig. 4). These results certified that the cause of death was RDS.

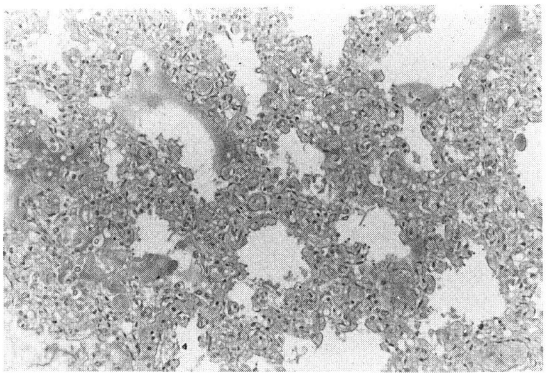

Fig. 4 (Case No. 4) Lung specimen showed no specific reaction at any site. (200×)

4 Discussion

4·1 On monoclonal antibodies and human pulmonary surfactant apoproteins: The first antibody used in this study was the monoclonal mouse antibody, which is specific to human pulmonary surfactant apoproteins. There are two types of monoclonal antibodies: PC6 and PE10. They do not react with human serum proteins or lung lavage of animals but only with human lung lavage and human amniotic fluid[5]. The two antibodies were confirmed to recognize both 36 kDa and 62 kDa protein groups, which were purified from human lung lavage[5]. They reacted strongly with the former and relatively weakly with the latter, and showed immunohistochemically the same results by immunoperoxidase staining[15].

The 36 kDa protein group is composed mainly of both 34 kDa and 37 kDa proteins, but it is considered to have also at least 6 isoforms from the results of isoelectric focusing electrophoresis[10,18]. The 34 kDa and 37 kDa proteins are glycoproteins which consist of a common 30 kDa core protein and different carbohydrate chains[17]. The monoclonal antibodies, PC6 and PE10, recognize different epitopes on the 36 kDa proteins, and the antigenic sites are not localized in the carbohydrate chain but in the non-collagen protein of the peptide [personal communication from Dr. Akino].

Consequently, except for 'pseudo-reactions' resulting from the endogenous peroxidase of granulocytes and monocytes or from non-specific background staining, the substances stained lightbrown in the sections by the immunoperoxidase technique were regarded as reaction products resulting from a specific combination between the monoclonal antibody and the antigenic proteins.

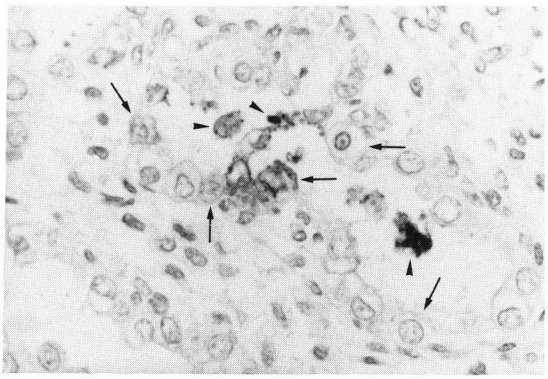

Fig. 5 (Case No. 31) The gestational period was estimated approximately as 20 to 22 weeks from all the measured values. Since the canalicular organization, which was formed from the cuboidal epithelial cells, was observed in the pulmonary tissue, the fetus was supposed to be still in the canalicular period of fetal pulmonary development. Very small amounts of the light-brown reaction products (arrow heads) were observed in the canaliculi. Some cuboidal epithelial cells (arrows) showed a positive reaction but none of the others did. The former was speculated to be alveolar type II cells and the latter, alveolar type I cells. (600×)

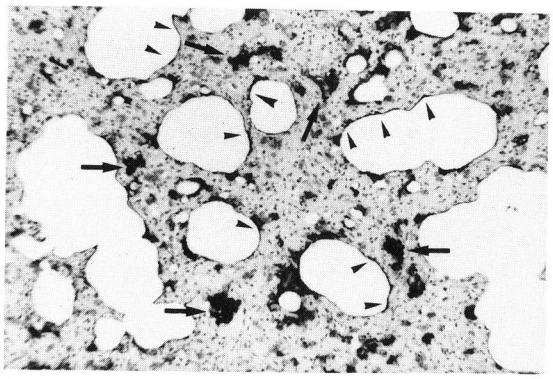

Fig. 6 (Case No. 22) The surface of alveoli which had not fully expanded, showed a linear positive reaction (arrow heads). Unexpanded alveoli contained masses of reaction products (arrows) in their lumen. (100×)

4·2 On the alveolar type II cells: Alveolar type II cells are relatively large and roughly cuboidal in shape, and their cytoplasm appears vacuolated[19]. By the immunoperoxidase method, the cytoplasm of the cells was stained light-brown. Under the observation by higher magnifying power, numerous fine reaction products of light-brown granules were found in the cytoplasm, and most of the granules were believed to be the lamellar bodies[4].

In the lung of the human fetus, alveolar type II cells are differentiated from the cuboidal epithelial cells in the second half of the canalicular period of fetal lung development[20]; therefore, they appear generally at 20 to 24 weeks of gestation. Then, after increasing in number and developing lamellar bodies, they begin to secrete surfactant into the alveolar lumen at 30 weeks of gestation at the latest[20-22]. After 34 or 35

weeks of gestation, the amount of secretion increases rapidly to prepare for alveolar expansion after birth[23].

Case No. 31 was found in a city sewage disposal plant. It was 26cm in height and 360g in weight and its placenta was 120g in weight. No signs of putrefaction were seen and the lapse of time after death was estimated to be a day or so. Hydrostatic tests of the lungs, stomach and intestines were negative. It was thought that no respiration was performed after delivery. The period of gestation was estimated to be about from 20 to 22 weeks from the measured values. The canalicular organization which was formed by the cuboidal epithelial cells was observed in the pulmonary tissue. The above findings suggested that the fetus was still in the canalicular period (17-24 weeks) of fetal pulmonary development[20]. Some light-brown colored reaction products were observed in the lumen of the canaliculi, though the amount was very small. Reaction products were also observed in certain numbers of the cubodial epithelial cells, though none were observed in any other cell types. The former cells were believed to be alveolar type II cells and the latter alveolar type I cells, though both types were still immature and morphologically undifferentiated (Fig. 5). This case presented convincing evdience that the secretion of surfactant begins from very early period, when the secreting epithelial cells are not yet sufficiently differentiated.

4·3 On postmortem alternation of the specific reaction:

4·3·1 Surfactant in the alveolar lumen: In the specimens of newborns and stillborns of optimal preservation the reaction products in the alveolar lumina were usually recognized as large masses, which were aggregations of numerous fine granules. The alveolar surface showed little reactions (Fig. 3). But as mentioned earlier, the staining profiles of the reaction products changed gradually as time passed.

In the specimens of 1 or 2 weeks after death, the surface of the alveolus was often coated linearly with light-browned reaction products, and occasionally the color was intense. This might indicate that apoproteins from the masses had spread on the alveolar surfaces and showed a linear positive reaction (Fig. 6).

In the specimens of 2 to 4 weeks after death, the reaction products were often seen in the degenerated products around the alveolar lumina. From this staining profile it was supposed that apoproteins in the lumina had been absorbed in the degeneration products and/or had infiltrated into them.

4·3·2 Surfactant within the alveolar type II cells: In most of the newborns and stillborns who were sufficiently matured and dissected approximately up to 3 days after death, a large amount of reaction products was recognized generally in the cytoplasms of the alveolar type II cells as well as in the alveolar lumen.

In the cases of mechanical asphyxia, however, there were some exceptional findings. For example, in case No. 15 the staining density of the alveolar type II cells was extremely thin, and the staining profiles and the amount of reaction products in the alveolar lumian were normal, though dissection were performed up to 3 days after death. In Cases No. 12 and No. 16, almost none of the type II cells showed positive reactions. Specimens from these cases revealed numerous roughly cuboidal or round shaped cells without any positive reaction. The cells were thought to be alveolar type II cells from their shapes, localization and distributions. The origin of these unstained type II cells was presumed to be as follows.

A) Autolysis, which occurred within the alveolar type II cells after death, destroyed the

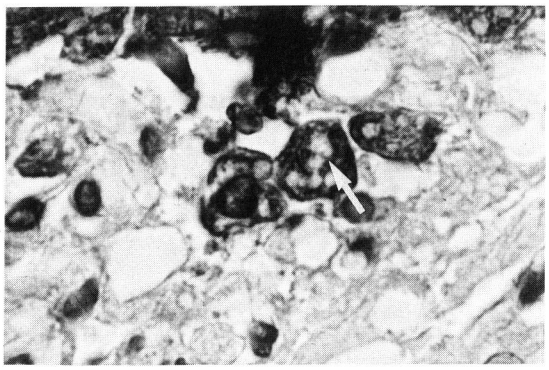

Fig. 7 (Case No. 22) Two roughly cuboidal shaped cells, which showed positive reaction, were standing side by side. The cells on the left still possessed a nucleus, though it was slightly deformed by degeneration. But other cells had denucleated already and showed a hole where the nucleus had been (white arrow). (1000×)

antigenic site of the apoproteins completely.

B) Little or no surfactant was stored in the type II cells at the time of birth, because after it was synthesized, it was secreted immediately into the alveolar lumina and none was stored.

C) Some stimulations which were given during the course of death caused the alveolar type II cells to release almost all of the stored surfactant.

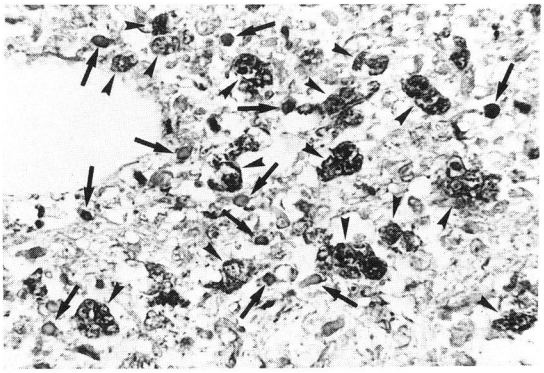

Fig. 8 (Case No. 30) The corpse was thought to be nearly or completely frozen and to have been well preserved; and though the tissue organization was slightly deteriorated. Some residual nuclei (arrows) were noted. Specific reactions were still observed (arrow heads). (400×)

A) is slightly possible as the reason but B) is doubtful. The most possible reason is C), and the stimulations are supposed to be alveolar hypoxia and/or the increase of surface tension. Since these profiles of unstained type II cells were not recognized in the stillborns but only in the infants who died of mechanical asphyxia, it should be considered that surfactant was released rapidly in response to alveolar hypoxia and/or the rising of surface tension in the course of asphyxia.

Degeneration tends to proceed more rapidly in alveolar type II cells than in other kinds of cells. This is probably due to the rapid and severe process of autolysis. As these secretory cells contain a large number of enzymes in their cytoplasms, it is possible that the lamellar bodies in their cytoplasm also change rapidly and are distintegrated, causing their reaction products to show diminished intensity. For the same reason, it appears that the cells change their shapes by denucleation or by further collapse. Actually in the specimens of approximately a week after death, both alveolar type II cells and light-brown 'doughnut shaped' cells were often encountered. Both groups of cells were thought to be alveolar type II cells, because the latter cells contained a round hole in the center where the nucleus had been localized, and their cytoplasm stained quite similarly to that of the intact alveolar type II cells surrounding the latter (Fig. 7).

4·4 On the detection of pulmonary surfactant: The specific reaction changed according to the postmortem changes. It was impossible to determine how long the specific reactions were able to detect, since the surrounding conditions in which the corpses were found, for example, indoors or outdoors, or in warm or cold seasons, differ among the cases (Table 3).

The specific reactions were detectable, however, for a surprisingly longer period of time, especially in the specimens of newborns or stillborns. It was very difficult to detect the type II cells in the specimens examined a month after death. But the reaction products remained in the alveolar lumen, though they decreased markedly in number. Moreover they were observed frequently at the sites where the type II cells were supposed to exist.

Even in the severely putrefied pulmonary tissues, in which tissue organizations could no longer be observed, traces of reaction products could still be detected. Considering the very high specificity of the monoclonal antibody and the normal serum treatment for the purpose of blocking non-specific reactions, the immunological reaction should be concluded to be a specific reaction to the apoprotein itself, and not a non-specific reaction to the degeneration products.

The reasons why the specific reactions were detectable for such a long time were proposed as follows. Firstly, the antigenic property of surfactant makes it relatively long lasting, since the matured lungs of newborn and stillborn contain a large amount of surfactant for breathing at the start of life. Secondly, it is supposed that the phospholipids which surround the apoproteins protect them from degeneration.

Accordingly the antigenic property does not readily disappear[14].

In conclusion, it was possible to detect reaction products in newborn and stillborn specimens up to about a month after death. On the contrary, if the specimen was aged 3 or more months after death, the products were very obscure or absent. And, if the lapse of time after death was more than 6 months, it was impossible to detect any reaction products.

There was, however, an exceptional case in which that the tissue organizations had been preserved relatively well and many specific reactions could be observed in the pulmonary tissues, though its lapse of time after death was estimated as 3 months or more. Case No. 30 was born by precipitated labor at term in November and its corpse had been kept in a toilet tank until the next March. Since the outdoor temperature remained near or below the freezing point all day long during this period, it was presumed that the corpse was nearly or completely fozen and thus had been kept under good preservation conditions (Fig. 8).

4·5 Consideration of the negative cases: Only 3 out of 39 cases showed negative results; two newborns which contained the RDS case, and one infant. In two cases except the RDS case the apoproteins were supposed completely decomposed due to highly advanced putrefaction.

Four possible causes of the negative results are postulated as described below.

A) Surfactant was completely absent at birth because it was not produced in the fetal lungs at all.

B) Surfactant had existed at birth, and then was completely used up, leaving no traces at the time of death.

C) Postmortem changes had dissolved the epitopes of apoproteins, leading to disappearance of the antigenic property.

D) There were some technical failures in the process of immunoperoxidase staining.

4·5·1 On the presumed cause A: When a fetus is aborted before 23 weeks of gestation, whether the abortion occurs artificially or spontaneously, no specific reaction can be detected in the fetal lungs at all[14]. Especially before 19 weeks of gestation, the presence of specific reaction cannot be proved, because the type II cells are not differentiated yet.

Whether in premature or in full-term babies, if surfactant was not secreted for some reasons at birth, for instance, the babies have no ability to secrete surfactant inherently, and thus no specific reactions could be found in their specimens at all. If the pulmonary tissues were autopsied within a short time after death, it is quite reasonable to consider that the babies were born with little or without any surfactant. In such cases, the newborn babies were suspected to have died immediately after birth without any respiration. It was also considered that they should be included in the group of cases which were regarded as stillborns or native death cases (e. g. respiratory failure).

4·5·2 On the presumed cause B: When the amount of surfactant stored in the alveolar lumen or in the alveolar type II cells is scarce at birth, or when insufficient complement of surfactant from type II cells is produced after birth, the newborn has difficulty maintaining expansion of alveoli. Consequently RDS must be conducted.

4·5·3 On the presumed cause C: The influences of postmortem changes should be considered carefully. Surfactant apoproteins in alveoli and in type II cells are thought to decompose gradually by autolysis or by putrefaction, and to lose their antigenic property.

As shown in Table 2, when the cases were autopsied within a week or so after death, it was possible to detect surfactant apoproteins in almost all cases, except for some particular cases such as case No. 4. The tissue structures of these cases were preserved well. When no specific reactions were observed, it was concluded that no surfactant had existed in the lungs of the victims at death, because it is not necessary to consider the disappearance of antigenic property due to decomposition.

In the severely degenerated tissues that had aged more than a month after death, if a negative specific reaction was found, the influence of putrefaction was considered first as a cause. But in these cases, it was quite difficult to conclude whether surfactant apoproteins were present at the time of death, or whether they had disappeared with the passage of time after death.

When specimens are examined several weeks after death, the influences of postmortem changes should be taken into consideration. In particular cases, the absence of specific reactions was noted in tissues which were relatively well preserved, though a specific reaction was clearly present at the time of death. It is possible that the newborn had less than the normal amount of surfactant at the time of death, which led to the rapid disappearance of apoproteins. Such cases require careful evaluation of whether or not surfactant was present before death.

4·5·4 On the presumed cause D: The problem of technical failures, for instance, the use of inadequate antibodies, can be ruled out in the present investiation.

4·6 Application to legal medicine: Immunoperoxidase staining, combined with the use of the monoclonal antibody and the results of HE staining, is a useful technique to confirm the cause of death of newborns.

4·6·1 Judgment of alveolar expansion: Expanded alveoli of the newborn indicate that the baby was born alive and that he breathed undoubtedly. By hematoxylin-eosin staining, it can be judged whether the alveoli was expanded or not. But another staining applied with the monoclonal antibody to the apoproteins is a valid double check of the judgment. The lungs of mature fetuses of over 35 or 36 weeks of gestation contained a large amount of stored surfactant in the unexpanded alveoli (Fig. 3). On the other hand, in the alveoli which had once been fully expanded, there were almost no large masses of reaction products observed (Fig. 1). One explanation is that most of the surfactant had been consumed to expand the alveoli, and another is that they had been released together with amniotic fluid into the air ways. Both explanations may be valid. Another interesting observation was the small masses of reaction products that were sometimes observed adhering to the walls of alveoli that were only halfway expanded.

Based on these findings, it was concluded that surfactant staining could be used to judge whether the alveoli had remained unexpanded or had once expanded but collapsed later. Keratin staining is often useful for this purpose, since alveoli that once expanded contain no keratin fibers, as they are released together with amniotic fluid. However, surfactant staining is more advantageous, since surfactant is distributed more widely and uniformly than keratin fibers, the number of individual variations is fewer, and moreover, the technique reflects the expansion of alveoli very well.

4·6·2 Evaluation of maturity of lungs: Anatomical maturity of pulmonary tissue can be evaluated by the observation of HE-stained sections with light microscopy[14,19]. Functional maturity, in other words, the ability of gas exchange in respiration, can be evaluated by the immunoperoxidase technique, though the technique reflects only some part of this process. The whole process cannot be estimated because immunoperoxidase staining cannot show the maturity of type I cells or the alveolar vessels, though it can demonstrate the function of lungs as gas-exchangers.

As mentioned before, adequate amounts of surfactant corresponding to the gestational period are detectable in normal fetal lungs after the middle of 20s weeks of gestation[19,23]. Particularly, a large amount of surfactant should be present in the alveolar lumen after 35 weeks of gestation[23]. If less than the normal amount of surfactant for anatomical maturity is found, developmental delay of the respiratory function can be judged. Thus the general maturity of the lungs can be determined rather accurately by immunoperoxidase staining.

4·6·3 RDS cases: If the pulmonary tissues are obtained as soon as possible after death and they show few postmortem changes, and if there is little or no specific reaction observed in the specimens, it should be

suspected that the baby died of RDS.

RDS can be suspected in cases that were often judged as stillborn, native death from respiratory failure of that of unknown causes, or mechanical asphyxia of unknown means. Considering that RDS is the major cause of neonatal death[24], many of RDS cases might have been imcluded in the 'out-of-hospital-death' cases in which the causes of death were unknown.

5 Conclusion

The results of this investigation confirm the usefulness of the immunoperoxidase technique using the monoclonal antibodies to clarify the cause of death, particularly in newborns, stillborns, and infants.

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References

1. Goerke, J.: Lung surfactant. **Biochem. Biophys. Acta 344**, 241-261 (1974).
2. Akino, T. and Ohno, K.: Phospholipids of the lung in normal, toxic and diseased states. **CRC Crit. Rev. Toxicol. 9**, 201-274 (1981).
3. Fisher, A. B. and Chancler, A.: Lung surfactant —phospholipids and apoproteins. **Exp. Lung Res. 6** (3-4), 171-174 (1984).
4. Williams, M. C.: Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. **J. Cell Biol. 72**, 260-277 (1977).
5. Kuroki, Y., Fukuda, Y., Takahashi, H. and Akino, T.: Monoclonal antibodies against human pulmonary surfactant apoproteins: specificity and application in immunoassay. **Biochim. Biophys. Acta 836**, 201-209 (1985).
6. Sawada, H., Yamabayashi, H. and Okajima, Y.: Isolation of apoprotein from bovine lung surfactant material by sodium deoxycholate. **Biochim. Biophys. Acta 493**, 196-200 (1977).
7. King, R. J.: Pulmonary surfactant. **J. Appl. Physiol. 53**, 1-8 (1982).
8. King, R. J.: The surfactant system of the lung. **Fed. Proc. 33**, 2238-2247 (1974).
9. Sawada, H. and Kashiwamata, S.: Sodium dodecyl sulfate—disc gel electrophoresis patterns of bovine lung surfactant. **Biochim. Biophys. Acta 490**, 44-50 (1977).
10. Sueishi, K. and Benson, B. J.: Isolation of maior apoprotein of canine and murine pulmonary surfactant: Biochemical and immunochemical characteristics. **Biochim. Biophys. Acta 665**, 442-453 (1981).
11. Sueishi, K., Tanaka, K. and Oda, T.: Immunoultrastructural study of sufactant system. Distribution of specific protein of surface active material in rabbit lung. **Lab. Invest. 37**, 136-142 (1977).
12. Williams, M. C. and Benson, B. J.: Immunocytochemical location and identification of the major surfactant protein in adult rat lung. **J. Histochem. Cytochem. 29**, 291-305 (1981).
13. Katyal, S. L. and Singh, G.: An immunological study of th apoproteins of rat lung surfactant. **Lab. Invest. 40**, 562-567 (1979).
14. Jaskoll, T., Phelps, D., Taeusch, W., Smith, B. and Slavkin, H.: Localization of pulmonary surfactant protein during mouse lung development **Dev. Biol. 106**, 256-261 (1984).
15. Kuroki, Y., Dempo, K. and Akino, T.: Immunohistochemical study of human pulmonary surfactant apoproteins with monoclonal antibodies. **Am. J. Pathol. 124**, 25-33 (1986).
16. Ng, V. L., Henderson, V. L., Mendelson, C. R.

and Snyder, J. M.: Characterization and properties of rabbit surfactant associated proteins. **Biochim. Biophys. Acta 754**, 218-226 (1983).
17. Floros, J., Phelps, D. S. and Taeusch, H. W.: Biosynthesis and in vitro translation of the major surfactant-associated proteins from human lung. **J. Biol. Chem. 260**, 495-50 (1985).
18. Katyal, S. L. and Singh, G.: Analysis of pulmonary surfactant apoprpteins by isoelectric focusing. **Biochim. Biophys. Acta 794**, 411-418 (1984).
19. Farrel, J.: Morphologic aspects of lung maturation in lung development, In: Farrel, P. M. (ed).: Biological and Clinical Perspectives. Vol. 1 13, Academic Press, New York (1982).
20. Kikkawa, Y.: Morphology and mophologic development of the lung, In: Scarpelli, E. M. (ed.): Pulmonary Physiology of the Fetus, Newborn and Child. 37-60, Lea & Febiger, Philadelphia (1974).
21. King, R. J., Ruch, J., Gikas, E. G., Platzker, A. C. G. and Creasy, R. K.: Appearance of apoproteins of pulmonary surfactant in human amniotic fluid. **J. Appl. Physiol. 39**, 735-741 (1975).
22. Katyal, S. L. and Singh, G.: An enzyme-linked immunoassay of surfactant apoproteins. Its application to the study of fetal lung development in the rat. **Pecliatr. Res. 17**, 439-443 (1983).
23. Kuroki, Y., Takahashi, H., Fukuda, Y., Mikawa, M., Inagawa, A., Fujimoto, S. and Akino, T.: Two-site "simultaneous" immunoassay with monoclonal antibodies for the determination of surfactant apoproteins in human amniotic fluid. **Pecliatr. Res. 19**, 1017-1020 (1985).
24. Behrman, R. E. and Kliegman, R. M.: Hyaline membrane disease (Idiopathic Respiratory Distress Syndrome), In: Nelson, W. E. (ed.): Textbook of Pediatrics (12th ed.), 336-371, Saunders, Philadelphia (1983).

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ヒト肺サーファクタントの免疫組織学的研究およびその法医学への応用

藤原正貴

肺表面活性物質（Pulmonary surfactant）は，肺胞の表面張力を低下させ，肺胞の虚脱を防いで肺胞を維持する．ヒトのサーファクタントは，約90%の脂質と約10%の蛋白質（アポ蛋白）の複合体である．34-37 kDaのヒト肺サーファクタント・アポ蛋白に対するモノクロナール抗体を使用した酵素抗体法(Immunoperoxidase staining)を用いて，司法解剖例の中から新生児，死産児ならびに乳幼児の肺組織に対して免疫染色を行なった．この結果から，周産期，特に早期新生児期におけるSurfactantの分布状態が明らかとなり，分娩後の呼吸の有無，その程度，時間などにより得られる特異反応像に差かあることがわかった．さらに死因，死後経過時間，肺組織の保存状態などの種々の要因によって特異反応像が変化することもわかった．業務上過失致死を疑われた事例において，本法を用いることで新生児呼吸窮迫症候群（Respiratory Distress Syndrome)による死亡であったことを確認できたので，本法の法医学への応用についての検討と併せて考察を行った．