Surface Tension, Metabolic Activity, and Lipid Composition of Alveolar Cells in Washings from Normal Dog Lungs and after Pulmonary Artery Ligation

IMPORTANCE OF A HIGHLY SURFACE-ACTIVE ACELLULAR LAYER

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ABSTRACT Lung-washings from mammalian species are a rich source of surfactant and of cells, predominantly alveolar macrophages, that could be important in the metabolism of the surfactant. We obtained washings from normal dogs, and from dogs that had had one pulmonary artery (PA) ligated 1 or 2 days earlier. Centrifugation of wash ($400 \times g$ for 20 min) separated a sediment, made up of cells at the bottom and a white layer, largely acellular, from the supernatant. The volume of sediment averaged 2.1 ± 1.4 ml, 75% of which was white layer. The cells resembled the large alveolar (type II) cells found in the lung; however they differed by at least one major histochemical reaction. The white layer had greater surface activity than the cells or the supernate, and was richest in phospholipids and lecithin. The cells lost their surface activity when rinsed and resuspended. These observations suggest that surfactant is normally present, mainly in an acellular fraction and possibly at the surface of the alveolar cells. The alveolar macrophages may either store surfactant, rather than synthesize it, or simply acquire a coat of surfactant during sedimentation. After PA ligation, the earliest abnormality was a decrease in the white layer; the cells were fewer, smaller, and weaker in metabolic activity.

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INTRODUCTION

Lavage of the lung through the bronchial tree yields fluid that has high surface activity and a lipid composition similar to that associated with surfactant (1–3). This lavage fluid also contains cells of alveolar origin, thought to be predominantly alveolar macrophages (4, 5). There has been little information about the possible relationship of these cells and of other components of lung washings to the metabolism of pulmonary surfactant.

In this study we collected lung-wash fluid from normal dogs and from dogs that had had unilateral pulmonary artery (PA) ligation 1 or 2 days before. This procedure has been a useful experimental model for the study of altered pulmonary surface activity (6–8). Our aims were to: (*1*) localize the distribution of surface activity in the supernatant and sedimentary fractions of the wash fluid from lungs; (*2*) correlate the lipid composition of these fractions with their relative surface activity; (*3*) assess the possible role of the alveolar cells in synthesizing or storing sur-

factant; and (*4*) examine the course of changes in the alveolar cell population and the surface activity of lung wash after PA ligation.

Alveolar cells which were recovered from washing normal lungs were rich in surfactant, and most of their surfactant content was at the surface rather than intracellular. The lung-wash sediment also contained an acellular white layer that contained a greater share of the total surface activity than the cells, and that was richer in total phospholipids and lecithin. After PA ligation, the earliest abnormality was a decrease in the white layer. There were also fewer cells and these lacked metabolic and surface activity.

METHODS

Experimental procedure. We conducted the experiments on dogs weighing from 9 to 27 kg and anesthetized with sodium pentobarbital (30 mg/kg). 31 dogs were used for obtaining normal samples. In these dogs, the lungs were "washed" for collection of alveolar cells, "white layer," and supernate by a procedure similar to that described by Myrvik and coworkers (4) and by Cohn and Wiener (5). In this procedure, approximately 200 ml (18 ml/kg of body weight) of Hanks' Balanced Salt Solution (BSS) was injected into each main bronchus (one at a time) immediately after the dog had been exsanguinated. Within 2–5 min the wash fluid was withdrawn by aspiration, aided by gentle kneading of the lungs. An equal volume of solution was introduced, and again withdrawn in the same manner.

The entire lung-wash fluid was recovered, which averaged 87% of the injected volume; it was then centrifuged in 1 500-ml container (i.v. infusion bottle) at 1500 rpm (approximately $400 \times g$) for 20 min at 4°C. The supernatant fluid was removed; 50 ml of it was saved for surface tension measurement and the remainder was used for lipid, phospholipid, and fatty acid analysis. In earlier experiments we treated the sediment as if it were comprised solely of cells. Later, recognizing that the sediment actually consisted of a brown layer of cells at the bottom and a white layer that was largely acellular on top, we made separate measurements of the two components.[1] These measurements were volume, surface tension properties, lipid and phospholipid composition, cellular morphology, and oxidative enzyme reactions. In some experiments we also obtained a total cell count (Fisher Autohemocytometer, Fisher Scientific Co., Silver Spring, Md.). Characteristics of the sediment, or of each of its two components, were compared with corresponding features of the supernatant fraction, using the paired *t* test.

PA ligation was performed in 18 dogs; the ligation was maintained for 2 days in 6 animals, and for 1 day in 12 animals. In these experiments, one pulmonary artery was ligated aseptically with two 00 cotton ligatures, and the dog was given penicillin and streptomycin postoperatively. 1 or 2 days later, the chest was reopened and both ligated and control lungs were sampled for histochemical and surface tension measurements. Each lung was then washed as described above for collection of sediment and its component parts. Data from the ligated lung were compared with data from the control lung in the same dog.

Histochemical techniques. Before sampling the lung for histochemical reactions and always before the washing, a small segment of lung was infiltrated with liquid gelatin by a tracheal catheter; the gelatin was allowed to congeal either by pouring cold saline or by applying an ice pack to the surface of the lung. A small (0.5–1 g) piece of lung was then snipped off that segment with sharp scissors, as a clamp was momentarily applied to prevent bleeding. Lung samples were immediately quenched in isopentane that had been cooled to −150°C with liquid nitrogen, and the samples later were sectioned in a cryostat[2] at −20°C. Gelatin infiltration insured that alveoli remained inflated until sectioned, and facilitated the sectioning of quick-frozen lung tissue (9, 10). The sections, 4–6 μ thick, were incubated in appropriate media for these oxidative enzyme reactions, using the principle of tetrazolium reduction: NAD and NADP-diaphorases, succinate, lactate, malate, glucose-6-phosphate dehydrogenases, and cytochrome oxidase (10). We selected these particular enzymes because they reflect the main metabolic pathways known to exist in alveolar cells (9–11). The same enzymatic reactions were determined on alveolar cells collected from normal and ligated lung and smeared on glass slides.

Surface tension determinations. Lung samples, 3 g each, taken before the washing were minced with fine scissors, "extracted" in 50 ml of 0.85% saline solution, strained through gauze, and placed in the trough of a modified Wilhelmy balance. The use of this balance, which measures surface tension as a function of extract surface area, has previously been described in detail (12). 50-ml of the original wash supernate was examined directly without further dilution. For determination of the sediment or of its fractions, the material to be examined was diluted in 50 ml of 0.85% saline solution (volume of the trough). None of the fractions was strained before being placed onto the balance. Comparisons were made between surface tension properties of the entire cellular fraction and the white layer from the same dog in seven animals, and between equal volumes of the two fractions in three other studies.

[1] The presence of two layers could not be distinguished when the wash was centrifuged in large, flat-based containers, but they were apparent when the sediment was resuspended in a small (10–15 ml) volume of 0.85% saline and the suspension recentrifuged, or when the original wash was centrifuged in 50-ml graduated tubes with a conical bottom. The white layer was pipetted out with a 9-inch capillary pipette.

[2] International Equipment Co., (Harris International), Needham Heights, Mass.

To find out how much surface activity of cells resided at the cell surface and how much of the surface activity was intracellular, we performed the following procedures: (*a*) cells were added to an inactive medium (e.g., BSS), then immediately removed by centrifugation and the tension of the cell-free medium was measured; (*b*) equal volumes of intact and sonicated cells (Sonifier,[3] model S-75, 12 bursts of 10 sec each, at 5°C) from the same animal were compared for surface activity in five experiments (disruption of the cells was verified by examining smears from the suspension); and (*c*) samples of cells were washed six consecutive times in BSS by resuspending them and repeating the centrifugation.

Biochemical analyses. The sediment from lung washings or its component layers was weighed to the nearest 0.1 mg and extracted for 24 hr in 20 times its volume of chloroform: methanol (2:1, v/v) (13). To achieve rupture of the cells and complete extraction of the lipids, the cells were sonicated in chloroform: methanol using a Branson Sonifier as described above. The dry weight of the cellular and white layers was determined by weighing a portion, drying it in an oven at 120°C for 48 hr and reweighing the samples. The supernatant portion of lung washings was lyophilized, weighed, and extracted with chloroform: methanol (13). These procedures extracted 97% (range, from 95 to 99%) of the lipid, as determined by reextraction of the residue for 5 days at 40°C and by comparison with the original extraction. Cholesterol and phospholipid were determined in aliquots of the chloroform extracts (14, 15). The phosphatide fractions were separated by thin-layer chromatography on silica gel and the lipid phosphorus was quantified (16). The fatty acid-ester composition was determined by gas-liquid chromatography after separation of these phosphatides by thin-layer chromatography (16). A Packard gas chromatograph with flame ionization detector was used and isothermal runs were made at 190°C using 6-ft columns packed with 20% diethylene glycol succinate. Quanitative results with N.I.H. fatty acid standards A, B, D, E, and F agreed with the stated composition data, with relative error less than 2% for each component.

RESULTS

Normal lungs

In 22 normal dogs, the mean volume of sediment upon centrifugation of the lung wash was 2.1 ± 1.4 ml. In the eight experiments in which the volumes of the cells and of the white layer were recorded separately, the white layer made up at least 75% of the total sediment.

An average of $59.7 \pm 14.6 \times 10^7$ cells were recovered from each dog. Most of the cells were about 20 μ in diameter; however, there was some variation in cell size. In size, shape, and relative intensity of oxidative enzyme reactions, the cells resembled the large alveolar cells and "free" alveolar macrophages seen in frozen lung sections from normal dogs. Among the histochemical reactions we tested, a notable difference was that the reaction for succinate dehydrogenase was moderately strong in the cell smears but weak or absent in the large alveolar cells in lung sections. Smears of the white layer showed relatively few cells, all of which were of the same type as those cells sedimenting in the bottom layer.

Surface activity of components of lung wash. Whole sediment had significantly higher total surface activity (lower minimal surface tension, $P < 0.05$) than lung extract, which, in turn, was significantly more active ($P < 0.01$) than the supernate nortion (Fig. 1). As little as 0.1 ml of the sediment gave a normal force-area curve on the surface balance.

In a group of seven experiments in which the total volume of cells and white layer from the same animals were compared, the white layer showed greater activity (minimal tension, 8.2 ± 3.1 as opposed to 13.7 ± 2.9 dynes/cm for cells, $P < 0.01$). However, no difference in surface activity was apparent in three instances wherein equal volumes of cells and white layer from the same dogs were compared.

Effect of washing the lung on its surface tension. The effect of the washing procedure described above on surface tension of lung tissue extracts was analyzed in nine studies. Extracts of

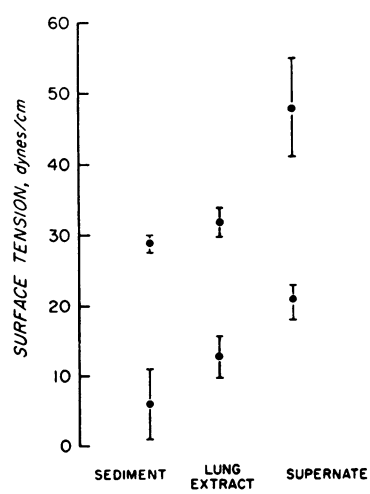


FIGURE 1 Mean and SD of minimal surface tension of whole sediment, supernate, and lung extract.

[3] Branson Instruments, Inc., Stamford, Conn.

TABLE I

Lipid Classes in Alveolar Cells, White Layer, Supernate, and Whole Sediment

	No. of determinations	Phospholipids	Cholesterol	Phospholipid/ cholesterol
		mg/g dry wt		
A Cells	5	161.80 ± 26.48	31.18 ± 5.01	5.33 ± 1.31
B White layer	6	501.40 ± 70.03	28.16 ± 6.75	18.67 ± 5.10
C Whole sediment	4	376.75 ± 104.31	38.98 ± 7.33	10.00 ± 3.90
D Supernate	10			12.26 ± 2.09
$P < 0.01$				B > D > A
$P < 0.05$				B > C > A

washed lungs gave higher maximal surface tension (36.2 ± 5.0 as compared to 31.8 ± 2.0 dynes/cm, $P < 0.05$) but showed the same minimal tensions as before washing.

Location of surface activity associated with alveolar cells. (*a*) Washing the cells repeatedly (six times) in BSS showed the loss of a major component of their surface activity (minimal tension of 11.3 ± 3.7 which increased to 19.1 ± 7.8 dynes/cm, $P < 0.05$. (*b*) The cell-free medium pooled from these washings exhibited considerable surface activity although the solution used for washing the cells was inactive to begin with (Fig. 2). (*c*) With each successive cell wash, an additional "white layer" appeared in the wash-fluid. The volume of these white layers decreased each time. (*d*) Sonification of the cells, performed on five occasions, failed to alter their surface activity. Similarly, sonification of the whole sediment from each of four other animals did not change the surface tension characteristics of whole sediment.

Lipid composition (Tables I, II, and III). These differences existed between whole sediment, cells, white layer, and supernate: (*a*) The white layer had a higher content of phospholipid (milligram per gram of dry weight) than the cells ($P < 0.01$). The phospholipid content of whole sediment fell between those of its two component parts. (*b*) In order to express the lipid content in the supernate and in the sedimentary fractions on a comparable basis, we used the phospholipid: cholesterol ratio in each fraction as a measure of the relative concentration of these two classes of lipids. On that basis, the white layer was the richest fraction in total phospholipids and the cells were poorest. (*c*) The relative concentration of lecithin (per cent of total phospholipid) was again highest in the white layer and lowest in the cells ($P < 0.01$). The per cent of lecithin was not significantly different from white layer, whole sediment, and supernatant. (*d*) Palmitic acid made up approximately 65% of the lecithin fatty acids in the white layer, 66% in the cells, and 64% in the supernatant. These values were not significantly different from one another.

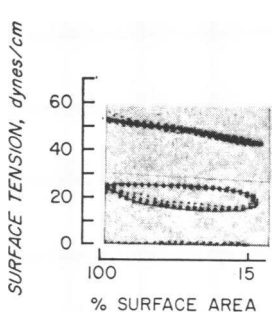


FIGURE 2 Surface tension–surface area relations of Hanks' Balanced Salt Solution (HBSS) (above) and of the same solution after it was used for one washing of alveolar cells.

Lungs after PA ligation

Decrease in volume of sediment, white layer, and cells. In the group of animals subjected to

TABLE II

Per cent of Lecithin in Total Phospholipids from Alveolar Cells, White Layer, Supernate, and Whole Sediment

	No. of determinations	Mean	SD	*P*
Cells	4	46.78	6.01	< 0.01
White layer	4	67.65	1.53	
				NS
Whole sediment	5	63.86	4.51	
				NS
Supernate	6	62.25	5.77	

NS, not significant.

TABLE III

Per cent of Palmitate in Lecithin Fatty Acids from Alveolar Cells, White Layer, and Supernate

	No. of determinations	Mean	SD	P
Cells	4	66.17	2.33	
				NS
White layer	5	64.59	1.90	
				NS
Supernate	5	63.67	7.36	

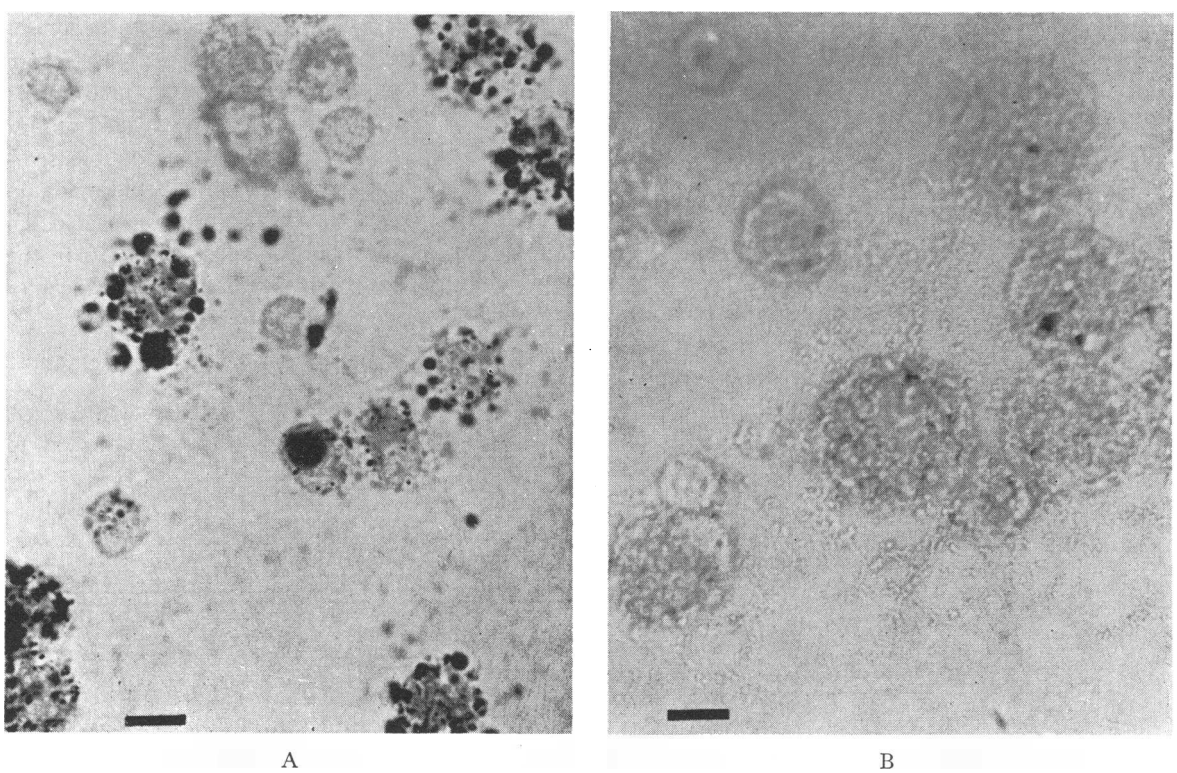
NS, not significant.

PA ligation for 24 hours, there was a significant decrease in the volume of total sediment recovered from the ligated lung, in relation to the control lung of the same dog (1.74 ± 0.78, decreased to 0.72 ± 0.51 ml, $P < 0.05$). An even more significant decrease in the volume of the white layer existed (2.08 ± 0.40 decreased to 0.77 ± 0.52 ml, $P < 0.01$, from another series of animals). There was no consistent change in the volume of the bottom (brown) layer of sediment, normally made of alveolar cells; however, it was often difficult to read that volume accurately due to admixture with red blood cells.

Changes in cell count and morphologic features. Lungs that had had PA ligation for 48 hr yielded fewer alveolor cells than did the control lungs. The cells were irregular in form and varied more widely in size, but most were smaller than 10 μ in diameter.

After PA ligation for only 24 hr, the cells were also smaller and less uniform in size and shape than normal cells. Total cell counts were obtained in only two instances; the count was reduced by 87% in one, and by 20% in the other.

Histochemical reactions. With PA ligation there was always a decrease in oxidative enzyme reactivity. This decrease was just as evident after 24 hr as it was after 48 hr. On the whole, of the reactions examined, NADP-diaphorase, succinate, malate, glucose-6-phosphate dehydrogenases, and cytochrome oxidase were most noticeably weak-



A B

FIGURE 3 Reaction for NADP-diaphorase in alveolar cells from washings of normal lung (A) and lung whose pulmonary artery had been ligated 2 days earlier (B), showing inhomogeneity of cell size and virtual absence of enzyme activity. (Both figures, × 1000; bar, 10 μ.)

TABLE IV

Relative Intensity of Oxidative Enzyme Reactions in Alveolar Cells after Pulmonary Artery (PA) Ligation

Enzyme	Intensity of reaction: Normal control	Intensity of reaction: After PA ligation
NAD diaphorase	++++	++
NADP diaphorase	++++	0 or +
Succinate dehydrogenase	+++	0 or +
Malate dehydrogenase	++++	0 or +
Lactate dehydrogenase	+++	+
Glucose-6-phosphate dehydrogenase	++	0
Cytochrome oxidase	++	0

ened (Fig. 3 and Table IV). In most cases, slight or moderate reactivity to NAD diaphorase and lactate dehydrogenase persisted after PA ligation, but in a few instances no reaction to any enzyme was apparent. There seemed to be a correlation between cell size and enzyme reactivity.

Impairment of surface activity. After 48-hr ligation of one PA, the surface activity of total sediment was reduced (surface tension, 27.8 ± 0.5, 7.0 ± 4.7, increasing to 39.2 ± 6.7, 21.0 ± 6.0 dynes/cm, $P < 0.05$). The reduction was apparent even when equal volumes of the sediment from control and ligated lungs were compared. In the 24-hr ligation dogs, there was no significant impairment in the surface activity of the total sediment or the cellular (brown) fraction, but the white layer was less active, volume for volume, than the normal controls (minimal tension, from 10.2 ± 5.7 up to 18.0 ± 3.8 dynes/cm, $P < 0.05$).

DISCUSSION

Distribution of surfactant in components of lung wash. The above observations show that lung-wash sediment from normal dogs is rich in surfactant, being considerably more surface-active than extracts of whole lung. Surface activity of the sediment is present mainly in a largely acellular, white layer, and, to a smaller degree, in a layer made up mostly of alveolar cells, predominantly macrophages. Upon repeated washing of the cells, they lose most of their surfacce activity; simultaneously, the surface activity of the washing medium increases and additional "white layers" appear with centrifugation. Furthermore, sonification of the washed cells does not yield more surfactant, as measured on the surface balance. It seems possible, therefore, that, in the intact animal, surfactant is normally "attached" or adsorbed to the surfaces of alveolar cells (and other cell surfaces) or is a part of these surfaces, and that with the washing of the lung and subsequent washing of the cells, surfactant is progressively removed from the cells until it can be found almost exclusively in the acellular fraction. This is not an inevitable conclusion, however; the data are consistent with other possible explanations. For instance, the alveolar macrophages may contain no surfactant at all during life, but merely may acquire a coat of surfactant as they are sedimented through the white layer.

In a paper published recently, Cavagna et al. (17) reported remarkably similar findings. They observed that the sediment from lung washings, but not the supernate, had high surface activity which could be removed by washing the sediment. They concluded that surfactant was adsorbed on the surface of the alveolar cells, could be desorbed by shaking and rinsing the cells, and then readsorbed onto washed cells. They did not, however, recognize the presence of an acellular, "white layer" component in the sediment. Among other reports dealing with examination of washings from mammalian lungs, the white layer was mentioned in only one (2).

Nature of the "white layer." The particularly high phospholipid content and phospholipid : cholesterol ratio in the white layer are consistent with the possibility that it is made up largely of cell membranes. This line of speculation would be in agreement with the observation that the white layer contains a large number of myelin forms (2). Myelin figures have also been demonstrated in an acellular layer that lines pulmonary alveoli (18) and such a lining could contribute to the white layer recovered in the wash.

Regardless of its exact structure and derivation, it is clear that the white layer is the fraction containing the largest proportion of surfactant from the lung. Abrams (19), working with saline extracts of lung, described a "pellicle" in the sediment after two centrifugations. This portion contained most of the surfactant and would appear

to correspond to the white layer in our experiments, except that the pellicle must be a more concentrated form of the surfactant; as little as 25 μg of the pellicle could lower surface tension to 12 dynes/cm, whereas the smallest amount of white layer that gave us such low tensions was 0.1 g. Even so, this is smaller than the amount of whole lung tissue possessing similar surface activity. The white layer is therefore sufficiently surface-active and its examination may be a simple, relatively sensitive index of pulmonary surface activity.

Correlation of surface activity with lipid composition. A correlation has previously been noted between total pulmonary phospholipids and extract surface activity in lungs of different animal species and in dogs after PA ligation (16). In this work, the white layer with the highest phospholipid content and phospholipid : cholesterol ratio had the greatest total surface activity of any component in the lung wash. The alveolar cells had a phospholipid content, phospholipid : cholesterol ratio, and a per cent of lecithin lower than the corresponding values for the white layer and the supernate, but which were close to those for whole lung tissue (13). The per cent of palmitate in lecithin was similar in the three components of lung wash. The relatively low surface activity of the supernatant portion might be attributable to a lower total content of surface-active phospholipids, rather than to the lack of a particular lipid. Morgan and coworkers (1), in a study of lung-washings from dogs, found that lecithin monolayers from the wash-supernate reached lower minimal surface tension than did lecithin from whole lung extract. They made no measurements on the sediment.

Role of the alveolor macrophages in metabolism of surfactant. The studies reported here suggest that alveolar macrophages may serve to store or to carry surfactant. The low surface activity of the washed cells and their relatively poor content of phospholipids, however, seem to weight against, although not exclude, the possibility of an additional role in the synthesis of surfactant. Such a role for these cells was considered unlikely by Elsbach (20) on the basis of the limited ability of these cells to form lecithin which carries two saturated fatty acids.

Identity and origin of the alveolar macrophage. The identity of the alveolar macrophage, particularly in relation to the large alveolar cell, has been examined by several authors (11, 21). The limited histochemical reactions we performed on smears of cells from the lung wash and on whole lung sections showed at least one major difference between the two cells: the macrophage gives a strongsuccinate dehydrogenase reaction, whereas the large alveolar cell is virtually nonreactive to this enzyme. Recently, Sorokin (11) has emphasized several differences in their morphologic and histochemical features.

The sharp reduction in the number of cells and total cell mass after PA ligation could be due to the interruption of the blood flow itself, a possible major source of alveolar macrophages (22), to the resultant focal atelectasis (23) rendering some alveolar units inaccessible to lavage, and to decreased availability of nutrients and precursors that may be required for the full development of these cells within the lung. The decrease in cell size and oxidative enzyme activity suggest that normal pulmonary arterial blood flow is essential for these cells to acquire or maintain their normal metabolic activity. In this regard, it is noteworthy that the enzymatic reactions whose activity decreased most were those involved in oxidative (Krebs cycle, cytochrome oxidase) and biosynthetic (NADP-diaphorase, pentose shunt) pathways.

Changes after PA ligation. The earliest and most significant measureable abnormality after PA ligation was a smaller volume of white layer and total sediment. In addition, there was a qualitative change, in that the surface-tension–lowering capacity of the sediment decreased, weight for weight, after PA ligation. This finding again underscores the importance of this fraction of the lung wash in relation to the total surface activity of the lung.

It is impossible to conclude as to whether the reduction in the cell mass contributed by itself to the decreased pulmonary surface activity, since the cells may merely carry a portion of the surfactant at their surfaces. The frequent unevenness in cellular size and metabolic activity was probably a reflection of the patchy distribution of morphologic changes in the lung after PA ligation (23).

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