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Loss of lymphocyte modulatory control by surfactant lipid extracts from acute hypersensitivity pneumonitis: comparison with sarcoidosis and idiopathic pulmonary fibrosis

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ABSTRACT: Surfactant components are recognized to exert a regulatory control on lymphocytes in physiological conditions, as testified by *in vitro* studies. However, what happens following lung injury has not been established. As surfactant composition is altered in interstitial lung diseases, this work was carried out to compare the modulatory impact of normal human alveolar fluids on lymphocyte proliferation, with that from inflammatory lung diseases which are characterized by distinct patterns of immunologically-mediated alterations (*i.e.* sarcoidosis, acute hypersensitivity pneumonitis, idiopathic pulmonary fibrosis).

Thymidine incorporation of allogeneic normal human blood lymphocytes was studied in the presence of total alveolar fluids or lipid extracts from 37 subjects, and phytohaemagglutinin (PHA) as T-cell mitogen.

The results show that: 1) total alveolar fluids and lipid extracts from normal subjects share a concentration-dependent suppressive activity on T-cell proliferation; 2) total alveolar fluids from diseased patients have lost this property, either by a lack of suppressive activity (*i.e.* idiopathic pulmonary fibrosis) or even by enhanced activity (*i.e.* sarcoidosis and hypersensitivity pnuemonitis); 3) lipid extracts from diseased patients still retain the suppressive activity of normal subjects, except for hypersensitivity; and 4) an imbalance in surfactant phospholipids with an increase in the inducers to suppressors ratio is more likely to explain this alteration in hypersensitivity pnuemonitis than changes in total lipid content.

In conclusion, alveolar lipid extracts from acute hypersensitivity pnuemonitis have lost the modulatory control normally exerted by surfactant lipids on lymphocyte proliferation *in vitro*. This alteration may contribute to the invasion of the lung by lymphocytes in acute hypersensitivity pnuemonitis *in vivo*.
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Surfactant is described as a surface tension-supporting material produced and metabolized by type II epithelial cells and essential for supporting the mechanical function of the lung. Alterations in amount, composition and/or function of surfactant components are the common hallmark of acute respiratory failure [1]. This field has been studied in interstitial lung diseases for several years now, and some qualitative and quantitative changes in surfactant components have been described in sarcoidosis, pulmonary fibrosis and pneumoconioses [2–7].

In all likelihood, this tension-active fluid, composed of phospholipids and surfactant associated proteins [8], possesses many other activities. For instance, surfactant is an important co-factor in bacterial and fungal phagocytosis, as well as in killing activities of macrophages [9–10]. In addition, it is involved in the immunomodulatory control of free cells in the deep lung (*i.e.* macrophages and lymphocytes). Recently, evidence has been increasing with regard to the lymphosuppressive effect of total alveolar fluids and surfactant extracts from bronchoalveolar lavages (BAL) obtained from normal human and animal lung [11–16]. This suppressive activity was demonstrated by impaired lymphocyte response to lectin mitogens and in allogeneic mixed lymphocyte cultures [11–16]. On the other hand, although surfactant phospholipids have been classified into suppressive and inductive subsets according to their *in vitro* activities [15], it remains to be demonstrated whether the physiological modulatory control of surfactant is disrupted in inflammatory lung diseases, such as those involving lymphocyte recruitment and activation in the lung.

The main hypothesis of this work postulates that a surfactant imbalance of phospholipid components might contribute to inflammatory processes in interstitial lung diseases through the loss of normal suppressive control of lymphocyte populations. Bronchoalveolar lavage fluids (BALF or total alveolar fluids) and (surfactant) alveolar lipid extracts were collected from normal volunteers and patients with active interstitial lung diseases (*i.e.* sarcoidosis, hypersensitivity pneumonitis and idiopathic pulmonary fibrosis). The influence of these products on normal blood lymphocyte proliferation was investigated in the presence of the phytohaemagglutinin (PHA) mitogen, a potent inducer of T-cell proliferation *in vitro*.

Materials and methods

Subjects

BALF were obtained from 37 adults recruited in the Service des Maladies Respiratoires et Réanimation Respiratoire (CHRU Nancy-Brabois, France) and l'Unité de Recherche Pulmonaire (CHU Sherbrooke, Quebec, Canada).

Nine subjects were nonsmoking healthy volunteers, referred to as normal, who accepted the BAL procedure (6 males and 3 females, mean age 29±3 yrs). Official ethical approval (Comité d'éthique de Lorraine) and informed written consents were obtained for all of the volunteers. All had normal physical examinations, chest X-rays and lung function tests. Occupational history did not reveal dust-related occupations. None of the volunteers were on medication or other drugs, and none had any acute viral or infectious respiratory disease for at least 1 month prior to time of BAL.

Twenty eight patients investigated for interstitial lung diseases were also included in the study: 1) 10 sarcoidosis (S) (stage 2 of chest X-ray classification), all had a biopsy-proven disease, and they were all nonsmokers; 2) 8 hypersensitivity pneumonitis (HP), all shared farmer's lung disease, with typical clinical history of acute disease, in addition to radiological, cytopathological and respiratory function characteristics, and all were nonsmokers, with two having quit smoking (5 and 10 yrs previously); and 3) 10 idiopathic pulmonary fibrosis (IPF), in whom no evidence for connective tissue disease, drug-induced pneumonitis, occupational exposure, mycobacterial infection or other granulomatous pulmonary disease was found, all showed typical clinical and respiratory function patterns, in addition to chest X-rays and computed tomography (CT) scan diffuse honeycombing, and biopsy-proven interstitial fibrosis, and all were nonsmokers.

None of the patients received any systemic or aerosolized steroid therapy.

Bronchoalveolar lavage (BAL)

BAL were performed according to the procedure detailed previously [17], using 5×50 ml aliquots of sterile 0.9% saline. The right middle lobe or the lingula was chosen as the site of lavage, according to radiological disease predominance. BALF were filtered through several layers of sterile nonsynthetic gauze to remove mucus, and centrifuged at 400×g for 10 min. Supernatants were divided into aliquots and stored frozen at -70°C for up to 6 months prior to assay. Overall, BALF was processed within 30 min of sample collection. Unprocessed aliquots were routinely controlled and cultured for aerobic and anaerobic bacterial contamination.

BALF processing

Cell-free BALF (35–70 ml) were concentrated tenfold by 48 h lyophilization, following by resolubilization and running through G25 chromatography columns (PD10, Pharmacia S.A, St Quentin en Yvelines, France). Columns were saturated with RPMI 1640 containing 25 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES) and 2 mM L-glutamine (Sigma Chimie SARL, L'Isle d'Abeau Chesnes, France) for desalting and osmolarity purposes, as described previously [17]. A similar procedure was performed on separate lipid fractions of each BALF following chloroform:methanol extraction [18]. A sample was taken from each total BALF and its surfactant extract counterpart in order to evaluate: 1) total phospholipid content in respective solutions by phosphorus assay [19]; and 2) for normalization of lipid content with RPMI 1640. Resulting solutions were then passed through a 0.22 µm sterile filter (Microstar, Costar Corp., Poly Labo, Strasbourg, France) and adjusted with heat inactivated 10% human A-B serum (hABS) from the same batch prior to their use on lymphocytes.

Surfactant phospholipid profiles

Major phospholipids were separated by thin layer chromatography on LK5D Silica gel 105A plates (Whatman Inc., Prolabo, Paris, France) in order to establish respective profiles of alveolar surfactant both from healthy volunteers and from subjects with lung diseases [17]. Separation was performed using a chloroform:methanol: water:triethylamine mixture. Following solvent migration, each sample was compared with equal amounts of standards (Sigma) identified by ninhydrin staining and scraped into borosilicate tubes for determination of phosphorus content. Stained phosphomolybdate complexes were read at 820 nm [20]. Individually tested phospholipids from each BALF were expressed as a percentage of total phospholipid content.

Preparation and culture of peripheral blood lymphocytes

Peripheral blood was obtained from healthy adult volunteers by venous puncture, defibrinated and layered 3:1 (v:v) over a lymphocyte preparation medium (Histopaque®1077, Sigma) in 50 ml polypropylene tubes

(Falcon, Becton-Dickinson S.A, Le Pont-de-Claix, France) [17]. After centrifugation at 500×g for 30 min, the cell layer was removed, washed twice in RPMI 1640 and reconstituted in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, 150 IU·ml^{-1} penicillin, 50 µg·ml^{-1} gentamicin and 10% heat-inactivated hABS at 1×10^6 per ml. This suspension was seeded in sterile 96 flat bottomed multiwell plates at 1×10^5 per well, before addition of the various agents. All blood samples used were controlled for differential cellular profiles and checked for any abnormality. Using this method, cells obtained usually contained more than 95% lymphocytes and less than 5% monocytes and polymorphonuclear cells by standard staining methods.

Assays on lymphocyte behaviour following alveolar fluid exposure

Experiments were performed in triplicate and blood lymphocytes from two different normal donors were systematically assayed. Whole BALF and lipid extract counterparts from normal and diseased patients were studied together at four different dilutions from a tenfold concentrate (100, 50, 10, 2%) by 60 µl aliquots in triplicate wells. Appropriate control wells were normalized with standard medium to a similar final volume. Phytohaemagglutinin lectin (PHA, I.B.F biotechnics, Villeneuve-La-Garenne, France) was chosen as a nonspecific T-cell mitogen, and added to each well (including reference controls) at an optimal concentration of 10 µg·ml^{-1} at the beginning of the experiments. Total length of each experiment was 96 hr, regardless of experimental conditions. Tritiated thymidine ([methyl-^{3}H]-thymidine, specific activity 6.7 Ci·mmol^{-1}, NEN, Dupont de Nemours S.A, Les Ulis, France) was added at a concentration of 1 µCi·well 16 hr prior to harvesting. Preliminary assays were performed to evaluate optimal PHA concentration- and experimental time-responses with and without BALF. PHA 10 µg·ml^{-1} as well as 96 h of assay time were chosen as optimal conditions for lymphoproliferation studies *in vitro*. Results of radiolabelled studies were expressed as a percentage of negative control values (standard medium plus 10% heat inactivated hABS and PHA, in the absence of alveolar fluid or lipid extracts) [17]. At the end of the procedure, cells from each plate were processed by a harvester (Harvester Skatron) on blotter paper, and the respective areas were left to dry in 6 ml vials (Mini polyQ, Soc Beckman Instruments, Gagny, France). Following addition of scintillation liquid (Ready Safe ™, Beckman, France), counts per minute (cpm) readings were performed using a β–counter.

Statistical analysis

Data were expressed as means±SD of the percentage of control values. Comparisons were performed using a Mann-Whitney U-test for nonparametric data. A value of $p<0.05$ was chosen as the threshold for statistical significance.

Results

Lymphocyte proliferation assays

Alveolar fluids obtained from the lavage procedures performed in several groups of healthy and diseased patients did not differ in volume return (normal 62±4%; S 63±2%; HP 56±6%; IPF 60±6%; NS). The range of phospholipid concentrations in alveolar lipid extracts was 2.5–340 µg·ml^{-1}. Average thymidine incorporation (±SD) into blood lymphocytes without PHA exposure was 931±447 cpm (within-assay variation between the two donors 34±7%; between-assay variation 136±35%). Average thymidine incorporation (±SD) into blood lymphocytes following PHA exposure (*e.g.* negative control) was 298,290±67,750 cpm (within-assay variation between the two donors 12.6±3%; between-assay variation 41.3±8%). There was a 410±82 fold increase of thymidine incorporation in lymphocytes exposed to PHA (negative control) compared to non exposed cells.

Whilst total alveolar fluids from control subjects revealed a concentration-dependent inhibitory activity of lymphocyte proliferation ($p<0.01$ at 100% dilution *vs* negative control), IPF fluids had lost this property. In addition, S and HP fluids contained a stimulatory activity for lymphoproliferation, which culminated at 10% dilution (S $p<0.05$ *vs* control; HP $p<0.01$ *vs* negative control) (fig. 1).

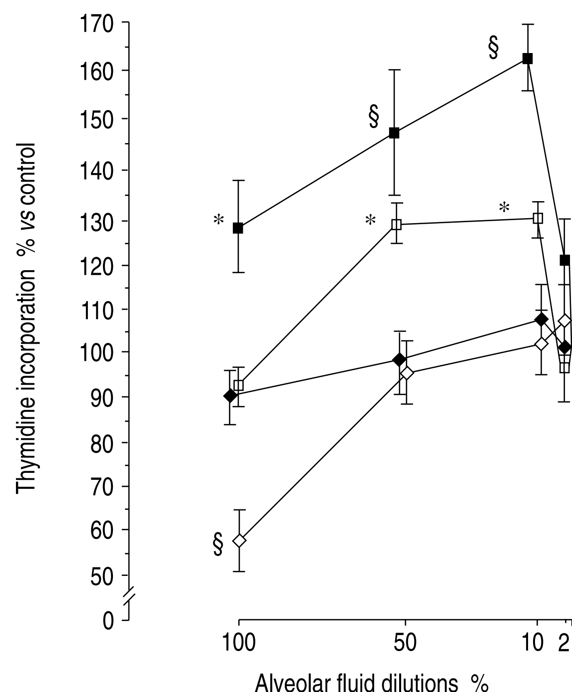

Fig. 1. – Modulatory activity of total alveolar fluids on lymphocyte proliferation. Data were obtained using tenfold concentrated human bronchoalveolar lavage fluid (BALF) and dilutions from 100% (undiluted BALF) to 2%. Values are expressed as mean percentages±SD of control values (culture medium + PHA without alveolar fluid). ◇: normal subjects; ◆: idiopathic pulmonary fibrosis; ❑: sarcoidosis; ■: acute hypersensitivity pneumonitis. Each BALF was tested twice in triplicate samples and in independent experiments. *: $p<0.05$ and §: $p<0.01$ *vs* control.

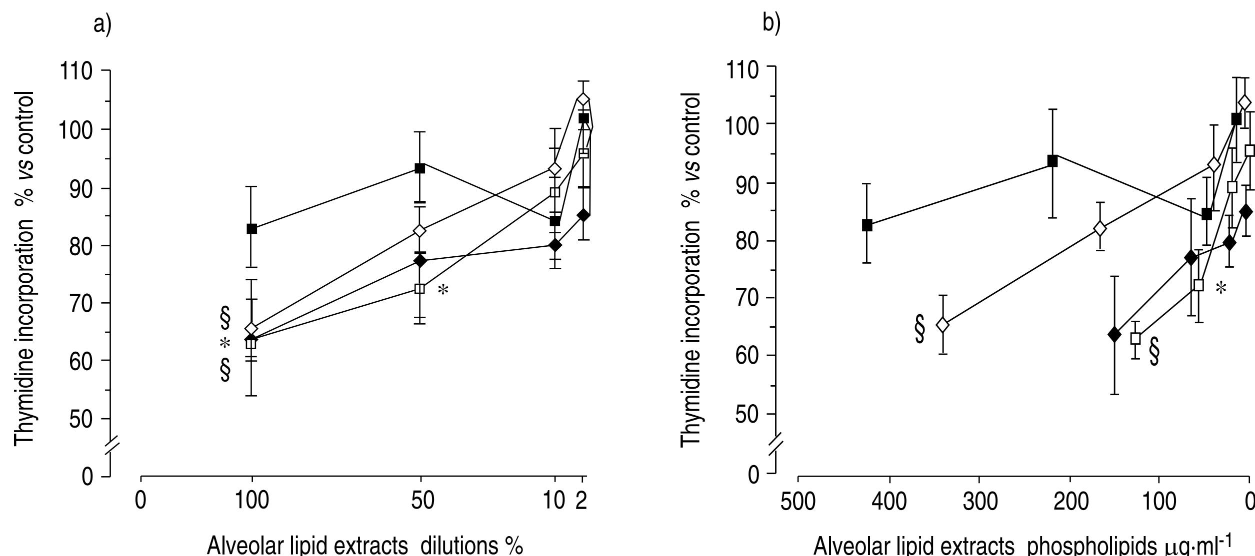

Fig. 2. – Modulatory activity of alveolar lipid extract on lymphocyte proliferation. a) Data were obtained using dilutions (100 to 2%) from tenfold concentrated human bronchoalveolar lavage fluid (BALF) where only the lipid fraction was used, and are expressed as mean percentages±SD of control values (culture medium + PHA without lipid extract). Each BALF was tested twice in triplicate samples and in independent experiments. b) effect of phospholipid concentration of alveolar lipid extracts. ◇ : normal subjects; ◆: idiopathic pulmonary fibrosis; ❑: sarcoidosis; ■: acute hypersensitivity pneumonitis. *: p<0.05 and §: p<0.01 *vs* control.

Lipid extraction of alveolar fluids revealed that the concentration-dependent modulatory activity was shared, at least in part, by surfactant lipids in control subjects, and was still retained in S and IPF extracts (p<0.05 *vs* negative control), but not by HP extracts (NS) (fig. 2a). Figure 2b shows that the surfactant lipid induced dose-dependent inhibition of lymphoproliferation was lost by HP extracts (fig. 2b)

Adding alveolar fluids or lipid extracts to lymphocytes more than 24 hr after PHA addition suppressed the inhibitory activity initially observed. On the other hand, adding PHA to lymphocytes more than 24 hr after alveolar fluids or lipid extracts did not alter the inhibitory effect (data not shown).

Surfactant phospholipid content and profiles

S and IPF shared a very low level of total phospholipids (S 11.8 µg·ml-1 and IPF 14.3±3.4 µg·ml-1, p<0.005 *vs* normal 34±4.1 µg·ml-1), whilst HP tended to have increased amounts of surfactant (42.7±5.4 µg·ml-1; NS) (table 1). Sphingomyelin (SM) as a proportion of total phospholipids was increased in all groups of patients; in addition, relative proportions of PC and PG lowered respectively in S and IPF whereas PE percentage increased in S (p<0.05 *vs* normal) (table 1).

Figure 3 shows several alterations of alveolar phospholipid content in S and IPF with a three to fourfold increase of SM, and a two to fourfold decrease of phosphatidylcholine

Table 1. – Surfactant phospholipid content and profile in normal and diseased human alveolar fluids

	Normal n=9	Sarcoidosis n=10	HP n=8	IPF n=10
Total phospholipids µg·ml-1	34±4.1	11.8±3.4§	42.7±5.3	14.3±3.4§
Phospholipid profile %				
Lysophosphatidylcholine	0.4 (0–3.7)	0.5 (0–16)	0.2 (0–0.8)	0.2 (0–0.5)
Sphingomyelin	1.3 (0–4.2)	13.1* (0.8–21.5)	12.4* (5.1–18.7)	13.7* (5.2–26.8)
Phosphatidylcholine	63.2 (51.7–80)	47.5* (43–50.2)	52.7 (34.5–68.9)	57.4 (43.7–68.7)
Phosphatidylserine + phosphatidylinositol	6.7 (0–15.1)	6 (0–18.1)	5.2 (3.1–7.9)	9.8 (0–18.3)
Phosphatidylethanolamine	5.8 (4.3–10.9)	11.6* (2.1–18.7)	8.1 (5.2–11.2)	6.5 (0–10.7)
Phosphatidylglycerol	18.5 (4.5–32.4)	21.1 (12–29.6)	16.3 (8.9–25.1)	12.2* (7.1–21.5)

Phospholipid values are means expressed as percentage total phospholipids. Ranges of values are shown in brackets. HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis. *: p<0.05 and §: p<0.005 *vs* normal.

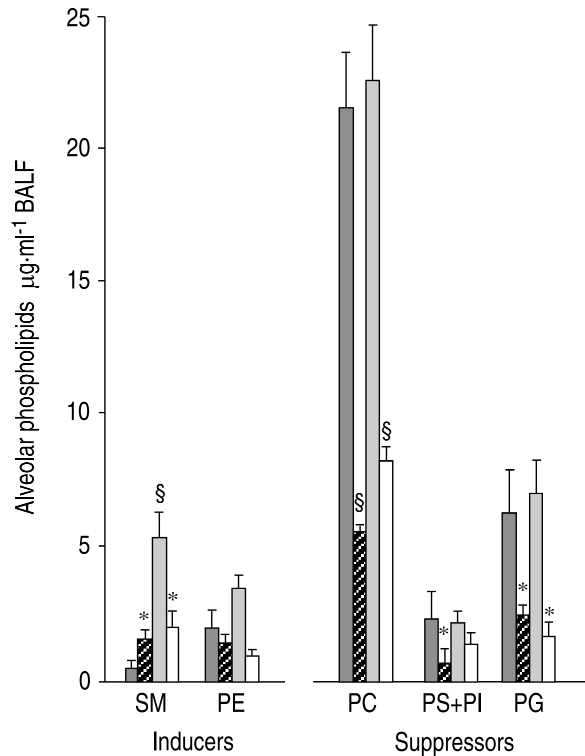

Fig. 3. – Amount and profiles of surfactant phospholipids. Data are expressed in µg of alveolar phospholipids·ml-1 BALF (mean±SD). Fluid recovery was similar in all studied groups. ▆ : controls; ▨ : sarcoidosis; ▭ : acute hypersensitivity pneumonitis; ▯: idiopathic pulmonary fibrosis. Phospholipids were separated into inducers (SM and PE) and suppressors (PC, PS+PI, PG) of lymphoproliferation; *: $p<0.05$ and §" $p<0.01$ *vs* normal. SM: sphingomyelin; PE: phosphatidylethanolamine; PC: phosphatidycholine; PS: phosphatidylserine; Pl: phosphatidylinositol; PG: phosphatidylglycerol.

(PC) and phosphatidylglycerol (PG) absolute values ($p<0.05$ *vs* normal). HP essentially demonstrated a tenfold increase of SM ($p<0.05$ *vs* normal), whilst other phospholipids remained within normal ranges (fig. 3). Phospholipids in figure 3 are expressed in two separate panels according to their recognized modulatory activity (*i.e.* suppressors *vs* inducers) on lymphocyte proliferation as demonstrated by WILSHER and co-workers [15].

Discussion

The present study documents and confirms the presence of a highly suppressive activity in alveolar fluids from healthy humans on lymphocyte activation and proliferation. A good portion of this activity is shared by surfactant phospholipid components of the epithelial lining fluid, and is likely to be concentration-dependent. Phospholipid profiles and content alterations observed in S and IPF did not modify this activity; whereas, a high increase of sphingomyelin (SM) with an imbalance in inducer/suppressor phospholipid ratio was probably responsible for the loss of this activity in acute HP.

The mechanisms behind the natural suppressive effect of surfactant have already been the subject of several studies. In this respect, ANSFIELD *et al.* [11] seemed to discard any cytotoxic mechanism, such as binding of mitogens by surfactant, inhibitory binding of thymidine or back regulation of deoxyribonucleic acid (DNA) synthesis. CATANZARO *et al.* [12] proposed an effect of surfactant during cell activation following receptor binding, since once a cell is committed to entry into the proliferative cycle, the surfactant has no longer a suppressive effect. WILSHER and co-workers [14–16] postulated that the inhibition of lymphocyte proliferation was due to associated alterations in cell membrane fluidity, attributed to changes in the membrane cholesterol to phospholipids ratio. On the other hand, it was demonstrated that several major phospholipids, purified individually, exerted either an inhibitory influence (*i.e.* PC, PG, phosphatidylinositol (P) and phosphatidylserine (PS) or a stimulatory influence (SM and phosphatidylethanolamine (PE)) on lymphocyte activation-proliferation *in vitro* [15]. Finally, SHIMIZU *et al.* [13] recently suggested that the inhibition of T-cell growth by surfactant was related to its inhibition of interleukin-2 production.

In the majority of studies on the modulatory function of surfactant components (or individually purified phospholipids) derived from normal lungs, it was reasonable to postulate that an imbalance of these components, following specific or nonspecific injury of the lung, might contribute to the maintenance of cellular activation and turnover. More precisely, imbalances in amounts of classified subsets of phospholipids might lead to a shift in the control of lymphocyte activation towards either an inductive or a more suppressive activity. To our knowledge, this is the first report in which diseased and normal alveolar fluids and surfactant are compared. Although we are aware that alveolar fluids recovered from BAL are composed of a myriad of biologically active molecules, they are, however, representative of the medium in which alveolar cells normally bathe. Thus, specific alterations of cellular activities induced by these fluids are indeed valuable for *in vivo* correlations.

Alterations in surfactant are recognized to be a consequence of lung injury, such as diffuse alveolar damage. However, with distinct initiating agents, many inflammatory lung diseases show different patterns of surfactant alterations: from normal or increased amounts (*e.g.* pneumoconioses [1, 6–7, 21]) to normal or decreased amounts (*e.g.* HP, sarcoidosis, IPF [1–5, 21–23]). More or less specific patterns described in the literature on interstitial lung diseases have delineated the variations of PG, PC, PG/PC, PG/PI and PC/SM as critical for the functional integrity of surfactant [15]. Present observations in the specific control exerted by surfactant on lymphocyte activation-proliferation suggest that the balance between the main phospholipids is more crucial than their concentration. SM which is greatly enhanced in acute HP (×10) and in acute respiratory distress syndrome (ARDS) [1, 24] is a component of cell membrane, released following toxic damage, while not naturally present in normal alveolar fluids [1, 23, 24]. It is also the most potent phospholipid in inducing a lymphoproliferative response to optimal PHA *in vitro* [15]. The role of SM *in vivo*, as suggested by our assays with *ex-vivo* alveolar products, is well-correlated with its *in vitro* function. However, alterations in SM proportions are not sufficient alone for

explaining the loss of influence of acute HP-derived lipid extracts on lymphocyte proliferation. Moreover, there is no clear correlation between absolute amounts of SM and their effect on thymidine incorporation.

Such a relationship between surfactant and lymphocytes must be added to the list of recently discovered dualities between alveolar epithelial cells, including their products and immunocompetent cells of the lung. Type II cells as main producers of surfactant phospholipids, are now recognized as possible active accessory cells capable of expressing human leucocyte antigen-DR (HLA-DR) II antigens [25], and also of reversibly blocking lymphoproliferation *in vitro* without any implication of surfactant products [26]. Thus, type II alveolar cells may immunoregulate lymphoid cells by two different mechanisms, and may contribute, along with alveolar macrophages, to the maintenance of homeostasis in the alveolar spaces.

In summary, it was demonstrated that an imbalance of inducer/suppressor surfactant phospholipids, such as that observed in acute HP, may be responsible for the loss of the physiological modulatory control of lymphocyte proliferation exerted by normal surfactant *in vitro*. An abundant accumulation of inducer SM is at least one of the prominent factors in acute HP. Although hydrosoluble cytokines contained in HP alveolar fluids largely contribute to the lymphoproliferative support observed *in vivo* [27], surfactant components may also take part in this network.

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