Chapter 3

Presence of reactive type II pneumocytes in bronchoalveolar lavage fluid

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Abstract

Objective
To evaluate the prevalence of reactive type II pneumocytes (RPII) in bronchoalveolar lavage fluid (BALF) samples obtained in patients with various pulmonary disorders.

Study design
Consecutive BALF samples were screened for the presence of RPII on May-Grünwald Giemsa stained cytocentrifuged preparations. BALF samples with and without RPII were compared with regard to prevalence, associated clinical diagnoses and cytological findings.

Results
RPII were generally large cells with a high N/C ratio and a deeply blue stained vacuolated cytoplasm. Most RPII occurred in cohesive cell groups, and the vacuoles tended to confluent. Cytological findings associated with RPII were the presence of foamy alveolar macrophages, activated lymphocytes and plasma cells. RPII were present in 94 (21.7%) out of 433 included BALF samples. Highest prevalences were noted in patients with systemic inflammatory response syndrome and alveolar hemorraghe. In addition, RPII tended to occur more frequently in ventilator-associated pneumonia, pneumocystis pneumonia, extrinsic allergic alveolitis and drug-induced pulmonary disorders. In contrast, RPII were not observed in BALF samples obtained from patients with sarcoidosis.

Conclusion
RPII were prevalent in about one-fifth of BALF specimens. They were mainly associated with conditions of acute lung injury and not observed in sarcoidosis.

Introduction

The microscopic examination of bronchoalveolar lavage fluid (BALF) is appreciated for various clinical applications. In our hospital, bronchoscopy with bronchoalveolar lavage (BAL) is routinely used in the assessment of interstitial lung diseases (ILD), ventilator-associated pneumonia (VAP) and opportunistic lung infections. For this purpose, a standardised protocol for laboratory work-up including a differential cell count on cytocentrifuged preparations has been implemented[1,2].

Type II pneumocytes are normally not present in BALF specimens, except when they are hyperplastic and shed into the alveolar space[3]. These so-called hyperplastic or reactive type II pneumocytes (RPII) were first observed in BALF samples obtained from patients with the acute respiratory distress syndrome (ARDS)[4,5]. Recently, we observed these cells in BALF samples of patients with drug-induced pulmonary toxicity[2] and pneumocystis pneumonia[6].
In the present study, we assessed the prevalence, the associated clinical conditions and the accompanying cytological findings of RPII in a series of consecutive clinical BALF specimens routinely submitted for analysis.

Materials and methods

Sampling technique

During a 36-month period (January 2000 – January 2003), all BALF samples obtained were considered. Bronchoscopy with BAL was performed with a fiberoptic bronchoscope (Pentax FB-15H/FB-15X, Pentax Medicals, Tokyo, Japan) and “wedged” into the right middle lobe or lingula (in the assessment of interstitial lung diseases) or the affected segmental or subsegmental bronchus (when pneumonia was suspected). The fluid was instilled into the subsegment through the biopsy channel of the bronchoscope in four aliquots of 50 ml sterile saline (0.9% NaCl, room temperature) and immediately aspirated and recovered. The BALF samples were transported to the laboratory within 15 minutes after collection and analysed within one hour upon arrival in the laboratory.

Laboratory processing

The volume of the recovered BALF was recorded. The first fraction, representing the bronchial fraction, was separated for mycobacterial culture and the remaining fractions were pooled for further analysis. The total cell count was performed in a Fuchs Rosenthal hemocytometer chamber. All BALF

samples were quantitatively cultured for bacteria and yeasts using 2- and 10-µl volumes transferred by pipettes[7]. They were also cultured for filamentous fungi and mycobacteria and, if clinically indicated, for *Legionella spp*. In addition, stains for detection of *Pneumocystis jiroveci* and filamentous fungi (Methenamine-silver stain), *Legionella pneumophila* (immunofluorescent antibody stain), acid-fast bacteria (auramine-rhodamine stain) and hemosiderin visualisation (Perls'stain) were performed on cytocentrifuged preparations. When community-acquired pneumonia was suspected, culture and serology for the detection of viral pathogens were done, and polymerase chain reactions for the detection of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* were performed.

Cytocentrifugation was done with the Cytospin 3 apparatus (Shandon Scientific, Ltd, Astmoor, U.K.) using uncoated precleaned slides (Menzel-Gläser, Emergo, Landsmeer, The Netherlands). The number of drops per cytospin chamber was adjusted to the total cell count[1]. Cytocentrifugation conditions were as follows: speed: 650 revolutions per minute, duration: 10 minutes, and acceleration rate: low. After drying, three preparations of each sample were May-Grünwald Giemsa (MGG) stained and subsequently sealed with a coverslip and mounting medium (Histomount, Shandon Scientific Ltd.). Six additional unstained slides were stored at –30^{o}C for further analysis.

Differential cell counts were made by examining 500 nucleated cells excluding squamous and bronchial epithelial cells. Cells were screened for the presence of intracellular organisms, and the number of cells with phagocytised organisms was expressed as a percentage of the 500-cell aliquot. All three preparations per BALF were screened for the presence of RPII cells, activated lymphocytes, plasma cells and foamy alveolar macrophages. RPII cells were identified by the descriptions reported by previous investigators[3,4].

Exclusion criteria

BALF samples were excluded if the retrieved volume was less than 20 ml, if the total cell count was less than 60.000/ml, or if the preparations showed excessive amounts of red blood cells, intercellular debris or damaged red blood cells.

Additional stains

From all samples in which RPII were observed as well as from 10 control samples, unstained cytocentrifuged slides stored at –30^{o}C were retrieved and stained by the alkaline phosphatase stain (Leucognost-alpa, Merck KGaA, Darmstadt, Germany) according to the instructions of the manufacturer. Ten

samples with RPII were stained with a peroxidase-antiperoxidase method using monoclonal antibodies against cytokeratin 7 and Ki-67 a monoclonal antibody that reacts with a nuclear antigen expressed in proliferating cells but not in quiescent cells (Dako, Glostrup, Denmark).

Clinical diagnoses – definitions

Diagnoses were based upon review of the patients' clinical records. Pneumonia was microbiologically defined as (1) recovery of an obligatory respiratory pathogen including *Legionella pneumophila*, *Mycobacterium tuberculosis*, Influenza virus, Respiratory Syncytial Virus (RSV), Adenovirus and *P. jiroveci*, (2) recovery of bacteria other than *L. pneumophila* and *M. tuberculosis* in quantities $\geq 10^4$ colony forming units/ml (except when only oral flora was recovered) and/or the presence of ≥2% cells with phagocytised organisms[8] . Infectious pneumonia was further categorised as community-acquired pneumonia, hospital acquired pneumonia (onset of pneumonia after ≥2 days of hospitalisation), or ventilator-associated pneumonia. All patients hospitalised at the Intensive Care Unit met the criteria for systemic inflammatory response syndrome (SIRS)[9], Diagnoses of interstitial lung diseases were made on pathological, radiographic and clinical findings. If these data were not available, the probable diagnosis as generated by means of a validated computer program was recorded[10]. This program uses demographic variables and BALF variables including the differential cell count in a polychotomous logistic regression model to predict the diagnosis of either infection, sarcoidosis, extrinsic allergic alveolitis and drug induced pulmonary toxicity, or idiopathic pulmonary fibrosis. The predicted diagnoses were considered if they displayed a ≥70% probability level. Further, a number of ≥20% hemosiderin loaden macrophages was considered as diagnostic for alveolar hemorrhage[11] Acute eosinophilic pneumonia was defined according to the criteria of Allen *et al.* which include also a BALF eosinophil count of at least 25%[12].

Statistical analysis

Comparisons of proportions between groups were analysed for statistical significance by means of the χ^2 test and differences in means were assessed by the student's t-test.

Results

During the study period, a total number of 489 BALF samples were submitted. Fifty-six samples were excluded from analysis, resulting in 433 samples obtained from 372 patients. The mean (±SD) age of the patients was 56±16 years (range 12–94 years), and the male-to-female ratio was 1.9: 1.

In 94 (21.7%) samples obtained from 87 (23.4%) patients, RPII were observed. Table 3.1 lists the clinical diagnoses corresponding to the BALF samples. From this table, it is clear that RPII were seen in a variety of pulmonary conditions. High prevalences of RPII were noted in patients hospitalised at the intensive care unit and suffering from SIRS and/or ventilator-associated pneumonia. Likewise, RPII were prevalent in nearly one third of BALF samples showing alveolar hemorrhage and also occurred in samples with (suspected) extrinsic allergic alveolitis and drug induced pulmonary toxicity. Although the total numbers per diagnosis were small, RPII were also observed in patients with pneumocystis pneumonia and eosinophilic pneumonia. In the group of intensive care unit patients with SIRS and in the group of alveolar hemorrhage, the differences reached statistical significance. In contrast, RPII were not observed in BALF samples obtained from patients with verified or suspected sarcoidosis ($p<0.05$) nor in patients with pulmonary tuberculosis.

Table 3.1 Occurrence of reactive type II pneumocytes in BALF samples obtained in patients with various pulmonary disorders[a].

Disorder	∑n[b]	n	%	p-value
Infectious disorder				
Community-acquired pneumonia (CAP)	38	6	15.8	N.S.
Hospital-acquired pneumonia (HAP)	22	3	13.6	N.S.
Intensive Care Unit (ICU) patients with systemic inflammatory response syndrome (SIRS) and ventilator associated pneumonia (VAP)	66	16	24.2	N.S.
ICU patients with SIRS, VAP not confirmed	77	26	33.8	0.007
Pneumocystis pneumonia	11	3	27.3	N.S.
Mycobacterium tuberculosis, pulmonary infection	6	0	0	N.S.
Interstitial lung diseases				
Sarcoidosis	23	0	0.0	0.020
Suspected of sarcoidosis	18	0	0.0	0.047
Drug induced pulmonary toxicity and/or extrinsic allergic alveolitis	20	6	23.1	N.S.
Suspected of drug induced pulmonary toxicity and/or extrinsic allergic alveolitis	8	3	37.5	N.S.
Idiopathic pulmonary fibrosis	15	2	13.3	N.S.
Suspected of idiopathic pulmonary fibrosis	22	1	4.3	N.S.
Other pulmonary diagnoses				
Acute eosinophilic pneumonia	4	2	50.0	N.S.
Alveolar hemorrhage	77	24	31.2	0.039
No final diagnosis	49	11	22.4	N.S.
Total[a]	433	94	21.7	

[a] Total number of diagnoses (n=456) outnumber the number of BALF samples (n=433), as combined diagnoses occurred: alveolar hemorrhage included cases of CAP, HAP and VAP (5 cases each), drug induced pulmonary toxicity (n=3), SIRS (n=2), tuberculosis (n=1) and idiopathic pulmonary fibrosis (n=1); [b] ∑n=total number of cases; n=number of cases with RPII cells in BALF, %=n/∑n x 100.

In most MGG-stained samples, RPII were already discerned at the low power field by their tendency to appear as cohesive cell groups. RPII were generally larger than the surrounding alveolar macrophages and their cytoplasm was more deeply blue stained. Intercytoplasmic connections were observed between the RPII in the cell groups. The volume of cytoplasm varied, but most RPII had a high nuclear/cytoplasmic ratio. Many RPII contained large vacuoles, that tended to confluent. Sometimes the vacuolated cytoplasm pushed the nucleus eccentrically towards the cell border. Nuclei were large and sometimes irregularly shaped. Although the majority of RPII occurred in aggregates, singly lying cells with corresponding morphologic features were occasionally observed. In some of these singly lying RPII, the cytoplasmatic vacuoles were smaller and more numerous (Figure 3.1, A-F). The various morphologic presentations of RPII were not linked to any particular clinical diagnosis (results not shown). From 86 samples, unstained slides were available for alkaline phosphatase staining, 75 (87.2%) samples with RPII showed positive cells clearly fitting the RPII morphology, no such cells were observed in the control samples without RPII cells. In the remaining RPII positive samples (n=11), very few RPII were seen on the MGG stained slides. The colour intensity of the positively stained cells ranged from faint to strong. All ten samples stained positive with both cytokeratin and Ki-67 markers.

With respect to the differential cell count, no differences were seen between BALF samples with and without RPII cells (Table 3.2). However, BALF samples with RPII cells displayed more frequently foamy alveolar macrophages, activated lymphocytes and plasma cells.

Table 3.2 Associated cytologic findings in BALF samples with reactive type II pneumocytes.

	Reactive type II pneumocytes present	not present	p-value
Differential cell count (mean % ± SD)	(n = 95)	(n = 338)	
Alveolar macrophages	42.8 ± 30.5	44.0 ± 34.7	N.S.
Lymphocytes	15.6 ± 19.9	16.4 ± 23.1	N.S.
Polymorphonuclear neutrophils	39.1 ± 33.2	38.1 ± 38.5	N.S.
Eosinophils	1.7 ± 5.3	1.3 ± 4.7	N.S.
Mast cells	0.5 ± 1.3	0.2 ± 0.7	0.096
Cytological findings (presence in numbers of BALF samples)			
Foamy alveolar macrophages	59	118	p<0.001
Activated lymphocytes	33	51	p<0.001
Plasma cells	11	16	p<0.05

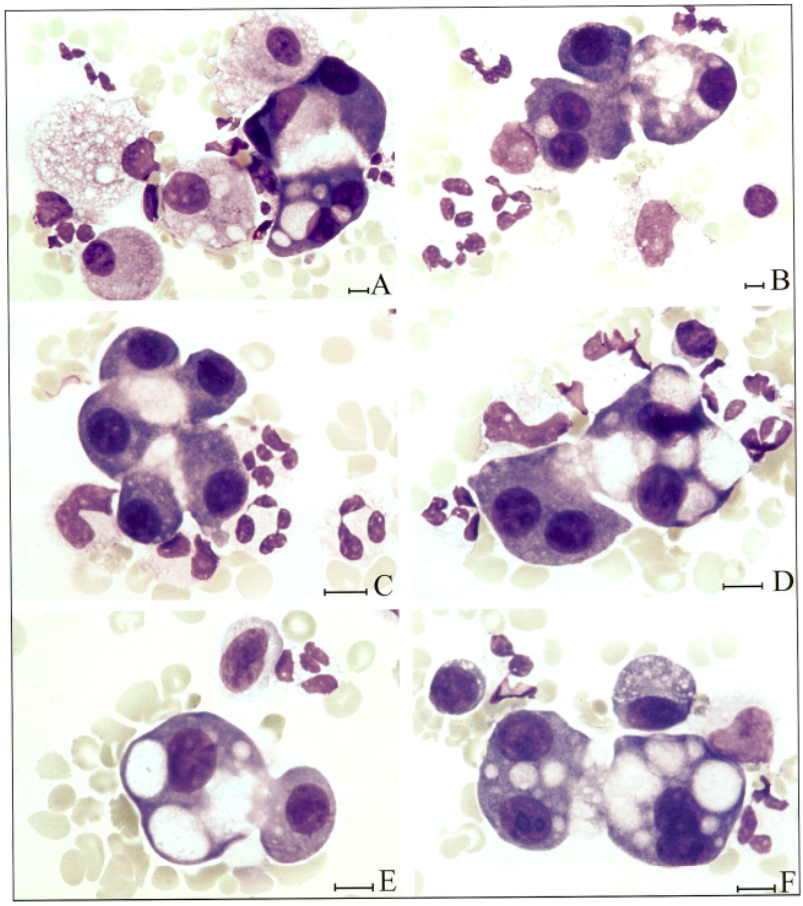

Figure 3.1 Reactive type II pneumocytes (RPII) in May-Grünwald Giemsa stained cytocentrifuged BALF samples. RPII showed a tendency to appear as cohesive cell groups. They were generally larger than the surrounding alveolar macrophages and their cytoplasm was more deeply blue stained. Groups of cohesive RPII cells were recognisable at the low power field (A and B). Intercytoplasmic connections were observed, and many RPII contained large vacuoles, that tended to confluent (C, D, E). The volume of cytoplasm varied, but most RPII had a high nuclear/cytoplasmic ratio. Sometimes the vacuolated cytoplasm pushed the nucleus eccentrically towards the cell border (A). Nuclei were large and sometimes irregularly shaped. Although the majority of RPII occurred in aggregates, singly lying cells with small vacuoles were occasionally observed (F). Scale bar represents 10 µm.

Discussion

In the present study, we observed RPII cells in 21.7% of a series of consecutive BALF samples. The alveolar epithelium is composed of squamous type I cells and cuboidal type II cells. Type II cells synthesise, secrete, and recycle all components of the surfactant that regulates surface tension in the lungs[13]. In addition, they contribute to epithelial repair by their ability to proliferate and differentiate into type I cells and into Clara cells, which are nonciliated cells in the terminal bronchioles[14]. Further, they play a role in alveolar fluid balance, coagulation and fibrinolysis[14].

Till now, only a few studies reported data on the prevalence of RPII in BALF samples. Stanley and coworkers observed RPII in 12 (31.6%) out of 38 samples obtained from patients with ARDS, while Ohori *et al*. reported RPII in only 10 out of 4 880 samples recovered from lung transplant patients[5,15]. In the latter study however, most bronchoscopies with BAL were performed as part of routine follow-up in patients who were not presenting symptoms of rejection nor infection. In the present study, the samples obtained in patients with SIRS and/or alveolar hemorrhage (which may comprise patients with ARDS) showed prevalences comparable to those in the former study. The overall high prevalence of RPII in the present series may in part be explained by our microscopy protocol, which asks for screening of three MGG stained preparations at the low power field in order to detect squamous epithelial cells (to rule out oropharyngeal contamination in suspected pneumonia) and/or foamy alveolar casts (pointing to pneumocystis pneumonia)[1,6]. Indeed, screening of the cytocentrifuged specimens at the low power field allowed fast and easy recognition of RPII, especially when they occur in groups or clusters, and in some of the BALF samples RPII were observed only in one out of three cytocentrifuged preparations (results not shown).

In the present study, cells morphologically classified as RPII reacted with the alkaline phosphatase stain, which stains Clara cells and RPII cells[16]. The morphological appearance of RPII on the MGG stained slides varied, in line with previous descriptions[4,5,15,17]. This variation in morphology may reflect intermediate stages in the differentiation of RPII to type I epithelial cell[13] and may explain for the different intensities of the alkaline phosphatase stain in the present series. Further, the epithelial origin of the RPII cells was confirmed by reaction with monoclonal antibodies against cytokeratin 7 (a quite specific phenotype of type II epithelial alveolar cells) and they were demonstrated to be in the proliferative phase (being marked by Ki-67 monoclonal antibodies), as previously demonstrated on histological preparations obtained from patients with diffuse alveolar damage[18].

The observation of RPII in BALF samples was originally studied in patients with ARDS[4,5]. Maximum shedding of RPII occurred during the exudative phase at 4 to 10 days after the onset of ARDS, and their presence persisted through the organizing stage[5]. The presence of RPII along with extracellular amorphous material as observed in Papanicoulaou-stained BALF samples has been reported as the cytologic hallmark of diffuse alveolar damage for which the clinical entity includes acute interstitial pneumonia and acute eosinophilic pneumonia[19-21].

Apart from these conditions, we found high prevalences of RPII in BALF samples of patients with extrinsic allergic alveolitis and drug-induced pulmonary disorders, pneumocystis pneumonia and ventilator-associated pneumonia. These findings support the suggestion that atypical cells, previously reported in various other lung cytology preparations and obtained from patients with pneumonia, pulmonary infarction, radiation, cytotoxic chemotherapy and bronchiectasis, are in fact RPII[4]. We observed no RPII in BALF samples of patients with pulmonary tuberculosis or sarcoidosis and, to our knowledge, RPII have not been reported in these conditions. This may be explained by the intensity and localisation of the pathologic process, *i.e.* granulomatous diseases such as in sarcoidosis and tuberculosis have a more chronical course and the alveolar epithelium may be not so severely altered as in cases of acute lung injury due to the previously mentioned conditions. The absence of RPII in sarcoidosis may be added to the other BALF cytological characteristics of this disease[22].

The pathophysiological role of RPII remains unclear. In methotrexate-induced pulmonary toxicity, RPII were demonstrated to modulate inflammatory cell recruitment by releasing neutrophil, eosinophil and monocyte chemotactic activities[23]. Further, RPII produce profibrotic factors such TGF-β_1, platelet-derived growth factor and TNF-α, but it is not clear whether they contribute to pulmonary fibrosis[13].

Several authors emphasize malignancy as an important differential diagnosis of RPII[4,5,22,24]. In particular, bronchoalveolar carcinoma must be differentiated. Subtle morphologic cytologic characteristics in favour of either RPII or carcinoma have been listed[4,22,24], but these features have a considerable overlap[15] and are better evaluated on Papanicoulaou-stained preparations than on MGG-stained preparations[25]. Further, no single immunohistochemic marker accurately discriminates between both cell types, *e.g.* RPII may react with monoclonal antibodies against CEA and B72.3 (a marker for the tumor-associated TAG-72 antigen)[4,17]. Careful clinicopathologic evaluation is necessary to ensure accurate diagnosis[4,5,15]. With regard to the present study,

several arguments apart from the high prevalence argue for the nonneoplastic nature of observed cells. None of the patients with RPII presented with localised mass lesions suspected for primary or metastatic carcinoma, but in stead they presented with a rapid clinical course and a clinical picture of acute pulmonary damage. Further, all patients with interstitial lung diseases have been followed up for periods extending to one year (results not shown).

Although not specific, the presence of associated cytological findings such as foamy alveolar macrophages, activated lymphocytes and plasma cells was in line with our previous findings and may point to either extrinsic allergic alveolitis, drug induces pulmonary toxicity or pneumocystis pneumonia[2,6,26]. When combined to these and other cytologic findings[22], the presence of RPII may be of additional diagnostic value in the assessment of interstitial lung diseases.

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