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Analysis of pleural fluid in idiopathic spontaneous pneumothorax; correlation of eosinophil percentage with the duration of air in the pleural space

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Pleural fluid analysis was performed in patients with idiopathic spontaneous pneumothorax. The objective of the study was to define the cell differentiation, and part of the cytokine profile, in relation to the duration of pneumothorax.

In the 23 consecutive patients (19 men, mean age 34·2 years, 17 smokers), pleural fluid was obtained immediately after chest tube drainage (n=6), or during thoracoscopy (n=17). Cytospins were carried out, and supernatant analysis of the different cytokines was performed using sandwich ELISA. All concentrations were corrected for dilution.

The duration of the pneumothorax was correlated with the rise in eosinophil percentage (r=0·81, P<0·00001) in pleural fluid. RANTES, platelet-activating factor (PAF), and monocyte chemotactic protein-1 (MCP-1) were detectable but no relationship with eosinophils or duration of the pneumothorax was found. Granulocyte-macrophage colony stimulating factor (GM-CSF) and interluekin-8 (IL-8) were not detectable. Interleukin-5 (IL-5) concentration correlated with the eosinophil concentration (r=0·84, P=0·037) and the eosinophil percentage (r=0·68, P=0·005) in the pleural fluid.

Idiopathic spontaneous pneumothorax causes a time-related rise in the eosinophil percentage in the pleural space, which correlates with the level of IL-5.

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Introduction

Idiopathic or primary spontaneous pneumothorax (ISP) is defined as 'air in the pleural space, of which the cause is unknown' (1). The normal space between the pleural blades is about 20 μm, and contains a low-protein, clear and colourless fluid. The pleurae themselves are covered by a single layer of mesothelial cells, basement membrane, and layers of collagen and elastic tissue. In the caudal part of the normal mediastinal pleura, an accumulation of macrophages, pleuripotential mesenchymal cells, lymphoid cells and plasma cells surrounding a centric lymphatic channel or vessel is found (4).

Pleural lavage may be a future source of diagnostic yield, similar to the present-day bronchoalveolar lavage, but few data on the subject are available.

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To our knowledge, there are no human reference values for cell differentiation in the pleural lining fluid. In rabbits, 32% of the pleural fluid cells were mesothelial cells, 61% were mononuclear cells and 7% were lymphocytes (5). Normal dog pleural fluid contains 70% mesothelial cells, 28% mononuclear cells and 2% lymphocytes (6). In human pleural fluid following congestive heart failure (which is therefore a low-protein fluid), the cell differentiation consists mainly of lymphocytes and other mononuclear cells. The number of polymorphonuclear leucocytes is small (7).

Eosinophilic pleural effusion is defined by the presence of more than 10% eosinophilic granulocytes (eosinophils) in the pleural effusion (1). Although eosinophilic pleural effusion is a relatively unspecific phenomenon with no clear cause, it has especially been associated with blood or air in the pleural space (8–10). The pathogenesis of the eosinophilia, after the entrance of air into the pleural space, is unknown. Pleural fluid eosinophilia has been reported before in patients with air in the pleural space, but has not been related to the time for which the air was present (8–10).

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Several cytokines are related to eosinophilic accumulation (11,12), such as interleukin-5 (IL-5) (13–15), interleukin-3 (IL-3) (13), granulocyte-macrophage colony stimulating factor (GM-CSF) (13), eotaxin (16), monocyte chemotactic protein-3 (MCP-3) (17), MCP-4 (18,19), macrophage inflammatory protein (MIP-1α) (20), RANTES (regulated upon activation in normal T-cells expressed and secreted) (20–22), and platelet activating factor (PAF) (23). During the past few years the number of cytokines related to eosinophilic accumulation has increased rapidly.

The main aspect of the relationship of eosinophils to duration of pneumothorax might be to prevent re-expansion pulmonary oedema, as this is also thought to be related to the duration of the compressed lung.

The purpose of this prospective study was to define the cell differentiation in relation to the duration of the pneumothorax (air in the pleural space) in the pleural effusion of ISP patients, and to reveal part of its related cytokine profile.

Patients and Methods

Twenty-three consecutive patients with ISP were included in our study, following informed consent, from March 1996 to March 1997 (19 men, mean ± SEM age 34·2 ± 1·9 years, 17 (ex)smokers). The medical ethical committee of the hospital approved the study design. In the medical history, the exact time and date of the first complaints of the pneumothorax were recorded. All patients had an acute and distinct start of the symptoms, and could recall the exact date and hour. We considered the start of symptoms as the entrance of air in the pleural space. After the ISP, the pleural fluid was collected during thoracoscopy. All patients underwent this thoracoscopy under local anaesthesia, as a routine treatment modality. Six patients received a chest tube prior to the thoracoscopy. In these cases, pleural fluid was also collected at the time of the tube insertion. Both samples (via chest tube and during thoracoscopy) were compared and related to cases without prior drains. The pleural fluid was collected undiluted (when possible), or after pleural lavage with 30 ml saline 0·9% in cases of insufficient yield. In these cases saline was spread out over the pleural surface with a syringe and was removed by suction drain. Only the first collected samples of the patients were used in the cell and cytokine analysis, which means that in six patients the fluid was collected via the first chest tube after insertion and in 17 patients the fluid was collected during the thoracoscopy (in which case no prior chest tube was placed).

On the day of pleural fluid collection, serum urea, C-reactive protein, leucocyte count and differentiation, as well as eosinophil count, were measured in venous blood.

Pleural fluid was immediately stored at 4°C. Cytospins were performed within 2 h and were stored at − 20°C. The supernatant portions were stored at − 80°C. A standard May Grünwald Giemsa staining was performed. Immunocytochemistry was performed with the alkaline phosphatase anti-alkaline phosphatase method (Dakopatt) using the following antibodies: Leu4 (anti CD3), THB4 (anti CD21), EBM11 (anti CD68) and BMK13 (anti-eosinophilic major basic protein=MBP).

Supernatants were analysed using sandwich ELISA for the cytokines in consecutive patients; IL-5 (R&D, detection limit 5 pg ml^{-1}, n=20), IL-8 (PharMingen, detection limit 50 pg ml^{-1}, n=16), MCP-1 (detection limit 30 pg ml^{-1}, n=16), RANTES (detection limit 50 pg ml^{-1}, n=16), GM-CSF (PharMingen, detection limit 30 pg ml^{-1}, n=16), and PAF (RIA, Du Pont, detection limit 0·3 ng ml^{-1}, n=7). MCP-1 and RANTES ELISA were developed in our laboratory (24).

Because the amount of intrapleural fluid is unknown, dilution with poorly mixed 30 ml saline will be impossible to estimate. Therefore, only the concentration corrected for the dilution factor was used. This was done using the urea dilution method (25,26). Because this urea method has not been tested for pleural fluid we correlated the serum urea with the undiluted pleural fluid urea concentration of 10 patients with proven pleural exudates (six men/four women; two pulmonary embolisms, six malignant pleural effusions, two pleuritis without certain diagnosis; lactate dehydrogenase, albumen, cholesterol and urea were measured in pleural fluid and serum at the same time). This revealed a good correlation between blood and pleural fluid urea concentrations (r=0·99, P<0·0001; paired t-test: not significantly different). We considered the urea dilution method as reliable. Only patients with longer existing pneumothoraces could deliver undiluted pleural fluid. The concentrations of the cytokines and PAF were corrected for the dilution factor through use of the serum and pleural fluid urea concentrations in the following equation: (ELISA) × [(urea in serum)/(urea in pleural fluid)]=end concentration (25). The detection limit for urea was 0·08 mmol l^{-1}. Because of the great difference between samples diluted by saline, and undiluted samples, the absolute counts were not considered to be useful. The patients were arbitrarily grouped in days after the start of symptoms. A delay in treatment of up to 3 days (due to the weekend), however, played a role here.

Statistical analysis was performed with linear regression analysis in SPSS 6.1 for Windows.

Results

The mean cell counts and cell differentiation in the pleural fluid samples (corrected for the dilution) and the mean cell differentiations are shown in Table 1.

Fig. 1 shows the time-related rise in the percentage of eosinophils in relation to the other cells found in the pleural fluid (r=0·81, P<0·00001). Cells present on the first day after contracting the pneumothorax are predominantly macrophages and neutrophils. On days 4–7, the eosinophil count was higher than that of all other cells. The neutrophil concentration showed a minor increase over time.

In six patients, a chest tube was introduced prior to the thoracoscopy. The samples taken at the time of the chest tube introduction and those taken following thoracoscopy were compared to ensure that no differences were caused by the different sampling method. We found no difference in

TABLE 1. Cell differentiation in pleural fluid related to the duration of pneumothorax

Cells	Mean (SEM)				
Days after onset of ISP	Day 0–1	Day 1–3	Day 4–7	Day >7	All
Patients (*n*)	10	3	6	4	23
Neutrophils (%)	50·7 (8·4)	37·2 (14·5)	18·5 (13·2)	3·3 (1·7)	32·3 (6·4)
Basophils (%)	0	0·4 (0·3)	1·7 (1·0)	1·6 (1·2)	0·8 (0·4)
Mesothelial cells (%)	0·3 (0·3)	4·3 (3·4)	0·2 (0·2)	3·9 (3·9)	1·4 (0·8)
T cells (%) ($CD2/3^+$)	3·4 (2·2)	6·3 (3·4)	11·7 (4·3)	7·9 (3·5)	6·7 (1·7)
Macrophages (%) ($CD68^+$)	41·8 (7·5)	32·4 (12·5)	25·8 (10·2)	19·4 (14·7)	32·5 (5·17)
Eosinophils (%)	3·1 (1·1)	19·4 (6·6)	42·5 (12·6)	63·7 (11·3)	26·1 (6·2)
Eosinophils $\times 10^6 l^{-1}$	0·7 (0·4)	9·2 (7·0)	7·9 (2·4)	4·3 (1·6)	4·0 (1·1)
WBC $\times 10^7 l^{-1}$	2·5 (1·3)	7·5 (5·9)	1·2 (0·3)	0·8 (0·4)	2·5 (0·9)

The start of the pneumothorax is defined as the start of the symptoms.
WBC=absolute white blood cell count corrected for dilution; T cell=lymphocyte.

cell differentiation in samples taken at the same time via primary chest tube or primary thoracoscopy ($0{\cdot}23<P<0{\cdot}9$; two-tailed *t*-test). However, we did not use any samples of pleural fluid when a chest tube had already been present for some time, because of the theoretical mechanical damage to the pleurae caused by the chest tube.

Table 2 shows the mean concentrations of the cytokines in the different time cohorts. GM-CSF and IL-8 could not be detected. MCP-1, RANTES and PAF were detectable. The values were corrected for the dilution by use of the described urea concentration method. No correlation with the duration of the pneumothorax was found for MCP-1, RANTES and PAF. PAF ($n=7$) was only detectable in the lower regions of the assay (0–1·7 ng ml^{-1}). The mean concentration of PAF was 0·29 pg ml^{-1} ($\pm$ SEM 0·11). PAF was not related to the cell indices, nor to chest tube drainage. IL-5 correlated with the eosinophil concentration ($r=0{\cdot}84$, $P=0{\cdot}037$) from 24 h onwards. IL-5 also correlated with the percentage of eosinophils in the cell differentiation ($r=0{\cdot}68$, $P=0{\cdot}005$). The correlation between the duration of the pneumothorax, and log IL-5 was not significant ($r=0{\cdot}17$, $P=0{\cdot}06$). The IL-5 concentration rose following the start of the pneumothorax complaints (trend: $r=0{\cdot}48$, $P=0{\cdot}069$).

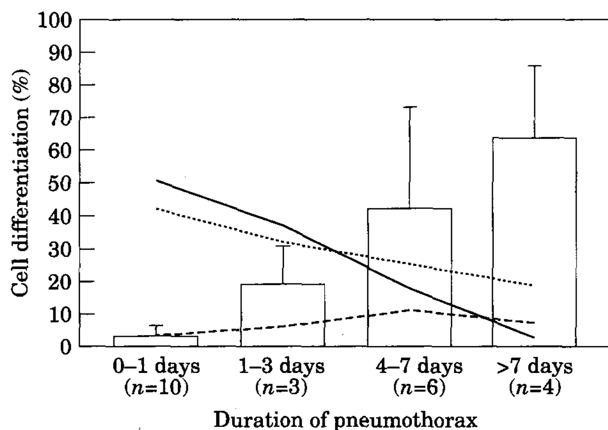

FIG. 1. Cell differentiation in pleural fluid after spontaneous pneumothorax; a time-related rise in eosinophils (□, eosinophils; ——, neutrophils; – – –, lymphocytes; · · ·, macrophages).

No elevations of C-reactive protein, leucocytes or eosinophil concentration (or percentage) in the serum were found at time of the pleural fluid sampling (data not shown).

Discussion

In ISP patients, by definition, there is air in the pleural space without any known underlying disease (1). ISP patients are usually able to define the exact time of contracting the pneumothorax because of the pleural pain accompanying the event. Our routine treatment of ISP (including the first event) is aimed at preventing recurrence, therefore a thoracoscopy under local anaesthesia is performed with talc pleurodesis. This routine approach makes it possible to investigate the pleural fluid of all referred ISP patients. Whether pleural fluid aspirated shortly after contracting the ISP is comparable to normal pleural fluid is not known because of the lack of reference values to normal human pleural fluid.

In rabbits and dogs, mesothelial and mononuclear cells are most common in the cell differentiation of normal pleural fluid (5,6). In the first 24 h after the start of the ISP in our study, we found predominantly macrophages and neutrophils and few mesothelial cells.

Our results show a clear relationship between the rise in the number (percentage) of eosinophils in the pleural fluid samples and the time after the start of the ISP (Fig. 1). To our knowledge, no systematic time-related rise in eosinophils in human pleural fluid has been previously described but the relationship between air and eosinophils in the pleura space has been reported (8–10). The pathogenesis is unknown.

Eosinophilic pleural effusion has been described in many diseases and is thought to be unspecific in relation to its cause.

TABLE 2. Supernatant cytokine analysis of pleural fluid at different times after the start of the first symptoms of the pneumothorax

Cytokines Days after onset of ISP	Mean (SEM)				
	Day 0–1	Day 1–3	Day 4–7	Day >7	All
IL-5 (pg ml^{-1}) (n=20)	2·1 (2·1)	70·3 (60·3)	29·5 (26·4)	63·4 (32·2)	28·4 (4·5)
RANTES (ng ml^{-1}) (n=16)	0·7 (0·3)	<LD	3·7 (2·4)	0·6 (0·4)	0·5 (3·0)
MCP-1 (ng ml^{-1}) (n=16)	6·6 (4·5)	0·9 (0·1)	0·5 (0·2)	1·2 (0·3)	3·4 (3·0)
GM-CSF (n=16)	<LD	<LD	<LD	<LD	<LD
IL-8 (n=16)	<LD	<LD	<LD	<LD	<LD

Concentrations were corrected for dilution.
IL=interleukin; RANTES=regulated upon activation in normal T cells expressed and secreted; MCP=monocyte chemotactic protein; GM-CSF=granulocyte/macrophage colony stimulating factor; <LD=less than the limit of detection of the assay.

Whether the rise in eosinophils is causally related to the pathogenesis of the pneumothorax is unclear. As eosinophils are not present immediately after contracting the pneumothorax, we do not believe that there is a causal relationship but consider them to be a consequence of air in the pleural cavity. Eosinophils may be important in the natural healing process of the spontaneous pneumothorax by inducing inflammation.

The rise in eosinophils is preceded by a high percentage of macrophages and neutrophils, which is an unusual beginning to the inflammatory process. In most inflammatory processes, neutrophilia is followed by mononuclear influx and the cause of the relatively high percentage of macrophages in the initial phase of pneumothorax is unclear. In a normal physiological situation, macrophages may already have been present before the pneumothorax occurred, or may have been present due to a prolonged inflammatory proces in the pleural space, and related to the inflammatory process present in the distal parts of the lung in patients with ISP.

In six cases, thoracoscopic sampling of pleural effusions was preceeded by chest tube insertion 1–3 days before. Analysis of the cells and cytokines revealed no differences between the patients with or without previous chest tube drainage in relation to the duration of the pneumothorax. Serial follow-up of these patients, i.e. two data points per patient, fits to the data we present here, although it was not included in the study design. We conclude that the chest tube did not influence the eosinophilic inflammatory process.

Several cytokines and chemokines are implicated in eosinophil accumulation. It has been suggested that lymphocytes are important in the accumulation process of eosinophils in the pleural cavity (27). Schandene described three patients with post-traumatic pneumothoraces, in whom $CD4^+$ T-cells were responsible for IL-5 production (14). In our study, however, T lymphocytes are only a minor population, suggesting that they were not involved in IL-5 production.

A significant correlation was found between the eosinophil and IL-5 concentration, suggesting that IL-5 is involved in the eosinophil chemotaxis. Eosinophils themselves, however, may also be a source of IL-5 (13). It is therefore unclear whether IL-5 is the cause or the result of eosinophil accumulation. In the literature, PAF, GM-CSF and Il-5 were shown to be important for the eosinophilic accumulation in the pleural cavity (13,23,28). We could not detect GM-CSF in either the diluted or the undiluted sample.

PAF is probably not involved, at least not in the pleural fluid, as the concentrations were 10-fold or more below the working range of the *in vitro* chemotactic assays (between 10 and 100 nmol).

RANTES has been reported to be involved, as an eosinophil chemoattractant (20–22). In our data, half of the consecutive samples had detectable RANTES, but with no significant relationship to the amounts of the different cells, the cell differentiation, or the duration of the pneumothorax.

Anthony *et al.* detected IL-8 in pleural fluids of patients with malignant pleural effusion (29). IL-8 could be measured in samples of pleural fluid after talcage (26), but not in the pleural fluid where air has been the only 'irritating' factor in the pleural space, despite the fact that this inflammatory process starts with a high percentage of neutrophils and macrophages. Therefore, another chemokine may be involved in the neutrophil accumulation in the first days after the presence of air.

MCP-1 was measurable in 14 of the 16 samples of ISP patients with different durations of pneumothorax. MCP-1 has been described as stimulating monocyte recruitment, especially in malignant pleural effusions (29). We could not detect a relationship with the absolute amount of monocytes, nor with the percentage of monocytes in the cell differentiation.

Several chemokines are thus shown to be detectable in the pleural fluids of ISP patients, which may give some direction to the pathogenesis of the pleural eosinophilia in case of air in the pleural space (in ISP patients). IL-5 was the only cytokine related to eosinophils. None of the other tested chemokines were related to the cell indices, which suggests that

other chemokines are involved in the inflammatory process in the pleural cavity.

The spontaneous pneumothorax patient who will undergo thoracoscopy seems to provide a good model for the investigation of eosinophilic pleural effusion. Future studies may include parietal and, when possible, visceral pleural biopsies to reveal more of the pathogenesis of this 'unusual' form of inflammation. For the daily practice, the detection of an eosinophilic effusion in a patient with ISP may be an indication of prolonged collapse of the lung. This can be important because rapid expansion of the lung might be followed by re-expansion oedema in these patients (30,31).

Conclusions

Human idiopathic spontaneous pneumothorax with air in the pleural cavity results in a time-related rise in the percentage of eosinophils in the pleural fluid. The eosinophil accumulation after 24–72 h follows an initial high percentage of neutrophils and macrophages. MCP-1, PAF and RANTES were detectable, but were not related to the duration of the pneumothorax, nor to cell indices. IL-5 correlates with the eosinophil concentration and percentage.

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