

Secretory leukocyte proteinase inhibitor is preferentially increased in patients with acute respiratory distress syndrome

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Secretory leukocyte proteinase inhibitor is preferentially increased in patients with acute respiratory distress syndrome. J.-M. Sallenave, S.C. Donnelly, I.S. Grant, C. Robertson, J. Gauldie, C. Haslett. ©ERS Journals Ltd 1999.

ABSTRACT: Inappropriate release of proteases from inflammatory and stromal cells can lead to destruction of the lung parenchyma. Antiproteases such as alpha-1-proteinase inhibitor (α_1 -Pi), secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (elafin) control excess production of human neutrophil elastase.

In the present study, the concentrations of α_1 -Pi, SLPI and elafin found in bronchoalveolar lavage (BAL) fluid from control subjects, patients at risk of developing acute respiratory distress syndrome (ARDS) and patients with established ARDS were determined.

Levels of all three inhibitors were raised in patients compared with normal subjects. SLPI was increased in the group of patients who were at risk of ARDS and went on to develop the condition, compared with the "at-risk" group who did not progress to ARDS ($p=0.0083$). α_1 -Pi and elafin levels were similar in these two populations. In patients with established ARDS, both α_1 -Pi and SLPI levels were significantly increased, compared to patients at risk of ARDS who did ($p=0.0089$) or did not ($p=0.0003$) progress to ARDS.

The finding of increased antiproteases shortly before the development of acute respiratory distress syndrome provide further evidence for enhanced inflammation prior to clinical disease.

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The acute respiratory distress syndrome (ARDS) represents a catastrophic form of acute lung injury that can be provoked by many diverse insults (*e.g.* multiple trauma, septicaemia, acute pancreatitis). This syndrome in its early stages presents all the characteristic features of acute lung inflammation, with a predominantly neutrophilic alveolitis [1] and resultant leakage of a protein-rich fluid into the interstitium and alveolar spaces. There is considerable evidence implicating neutrophils in the pathogenesis of ARDS.

Significantly, a potent neutrophil chemotactic factor, interleukin (IL)-8 is found in abundance in the alveolar airspace of ARDS patients, and high levels of IL-8 in the bronchoalveolar lavage (BAL) fluid of patients at high risk of developing ARDS are associated with progression to the full-blown disease [2, 3]. In addition, investigators have described elevated levels of neutrophil-derived products such as the proteolytic enzymes neutrophil elastase [1, 4] and collagenase [5, 6] in the alveolar space of patients with established ARDS. These enzymes have been im-

plicated as injurious agents in chronic lung diseases such as chronic bronchitis and emphysema in which an imbalance between human neutrophil elastase (HNE) and its physiological inhibitors has been postulated to result in parenchymal damage. Historically, alpha-1-proteinase inhibitor (α_1 -Pi) was regarded as the main physiological inhibitor of HNE, following the discovery of an inherited form of emphysema which develops in families homozygous for the Z allele of the α_1 -Pi gene [7]. However, more recently, two potent low molecular weight inhibitors of HNE, the secretory leukocyte proteinase inhibitor (SLPI) and the elastase-specific inhibitor (ESI), also called elafin, or skin-derived antileukoprotease (SKALP), have been characterized at both the protein and gene level [8–11]. There is now significant evidence that elafin and SLPI are important antiproteases in the lung, in both health and disease [12–15].

To date, no studies have investigated the presence, importance and activities of α_1 -Pi, SLPI and elafin in ARDS. The aim of this study was, therefore, to assess the potential relative importance of these inhibitors within the lung, both in patients in the early risk period for ARDS and in patients with established disease.

For editorial comments see page 947.

Materials and methods

Subjects

In this study, ARDS was defined using the American–European Consensus definition [16]. With regard to multiple organ failure, the extent of organ involvement and severity of organ failure were quantified with a modified Goris organ failure score as described previously [17]. Two patient groups were enrolled, first, a group of patients with conditions associated with a high risk of progression to ARDS ("at-risk" group, $n=24$; table 1), and, second, patients with established ARDS disease ($n=16$; table 2). At-risk patients were enrolled on initial hospital presentation and established ARDS patients were enrolled within 24 h of diagnosis. All patients were attending the Royal Infirmary or Western General Hospital, Edinburgh. Assays were also performed on eight normal control subjects. Informed consent was obtained from patients, or their relatives or guardians. This study was approved by the Lothian Health Board Ethics Committee.

At-risk patients came from two well-defined patient populations, namely severe multiple trauma who required intubation in the emergency room (MT, $n=16$) and patients with perforated large bowel undergoing laparotomy (PB, $n=8$), the sampling being performed preoperatively when the patients were under general anaesthesia. From this group, 10 patients subsequently progressed to ARDS (seven MT and three PB). The median age of these patients was 51 yrs (range 21–82 yrs). For MT patients, the estimated median time in minutes from the initiating insult to study enrollment was 120 min (range 90–240 min). While it is

Table 2. – Patient details for the group with established acute respiratory distress syndrome (ARDS)

Patient No.	Aetiology	Outcome	MOFS	$P_{a,O_2}/F_{I,O_2}$
1	Sepsis	Lived	2	24
2	Sepsis	Lived	5	12.7
3	Sepsis	Lived	3	16.4
4	Sepsis	Lived	7	16.5
5	Sepsis	Died	NA	NA
6	Sepsis	Died	6	13.7
7	Sepsis	Lived	2	17.8
8	Sepsis	Lived	2	22
9	Sepsis	Lived	6	18.2
10	Sepsis	Died	9	14.2
11	Sepsis	Died	8	14.8
12	Sepsis	Died	6	9
13	Sepsis	Lived	8	10.5
14	Sepsis	Lived	5	29
15	Sepsis	Died	6	13
16	Sepsis	Lived	6	16

MOFS: multiple organ failure syndrome; P_{a,O_2} : arterial oxygen tension; F_{I,O_2} : inspiratory oxygen fraction; NA: not available. MOFS and lowest $P_{a,O_2}/F_{I,O_2}$ (kPa) are those attained subsequently during the patients' hospital stay.

more difficult to ascertain the precise onset within the PB patient group, the median time of symptoms prior to study enrollment was approximately 24 h (range 6–72 h). All of the patients in this latter group satisfied the criteria for systemic inflammatory response syndrome (SIRS) [18]. The mean time between bronchoscopy and development of ARDS in the group of patients at risk of ARDS who developed the disease was 47.5 h (range 6–96 h).

Table 1. – Patient details for the group at risk for acute respiratory distress syndrome (ARDS)

Patient No.	Aetiology	TRS	ISS	Progressed to ARDS	Outcome	MOFS	$P_{a,O_2}/F_{I,O_2}$
1	Trauma	5.676	15	No	Died	1	17.8
2	Trauma	5.221	44	No	Alive	2	27
3	Trauma	7.108	33	No	Alive	1	18
4	Trauma	7.550	24	No	Alive	3	21
5	Trauma	5.970	29	No	Alive	1	45
6	Trauma	2.047	29	No	Died	1	22.3
7	Trauma	4.740	38	No	Died	0	42
8	Trauma	7.11	15	No	Lived	1	29
9	Trauma	4.44	29	No	Died	0	38
10	Perf. bowel			No	Alive	1	25
11	Perf. bowel			No	Died	2	27
12	Perf. bowel			No	Alive	0	29.5
13	Perf. bowel			No	Alive	0	25.8
14	Perf. bowel			No	Alive	0	NA
15	Trauma	2.628	59	Yes	Lived	5	9.5
16	Trauma	2.198	43	Yes	Died	6	11
17	Trauma	5.97	29	Yes	Lived	7	7.1
18	Trauma	7.841	27	Yes	Lived	2	24
19	Trauma	7.841	45	Yes	Died	1	22
20	Trauma	7.841	33	Yes	Lived	1	29
21	Trauma	7.841	29	Yes	Lived	2	10.2
22	Perf. bowel			Yes	Lived	8	10.5
23	Perf. bowel			Yes	Died	6	8.95
24	Perf. bowel			Yes	Lived	5	12.7

TRS: Trauma Revised Score; ISS: Injury Severity Score; MOFS: Multiple Organ Failure Score; P_{a,O_2} : arterial oxygen tension; F_{I,O_2} : inspiratory oxygen fraction; Perf.: perforated; NA: not available.

All enrolled patients with established ARDS ($n=16$) had sepsis as their initiating agent. The group median age was 49 yrs (range 18–75 yrs). The patients' demographic data are summarized in tables 1 and 2.

Bronchoscopy

A bronchoscopy and BAL were performed on enrolled patients. For the "at-risk" patients these procedures were conducted on initial hospital presentation and, in the case of patients with established ARDS, within 24 h of the diagnosis being made. The fiberoptic bronchoscope was introduced through an indwelling endotracheal tube. The distal end of the bronchoscope was wedged into the middle lobe. Three 60-mL volumes of saline (0.9% (w/v) sodium chloride solution) were instilled, then gently aspirated immediately. On average, 55% of instilled fluid was recovered (range 40–81%). All bronchoscopic procedures were performed by one doctor (S.C. Donnelly). Recovered fluid was stored at 4°C until processing within 1 h. After straining through sterile gauze, the fluid was centrifuged at $400 \times g$ at 4°C for 10 min to recover cell-free supernatant. BAL supernatant was respun at $1,000 \times g$ for 10 min at 4°C to remove cellular debris and stored at -70°C until assays were performed. Eight normal control subjects (two smokers, five females, mean age 23 ± 2 yrs) also underwent BAL.

Enzyme-linked immunosorbent assay for the measurement of antiproteinases

The concentration of α_1 -Pi was determined as described previously [15]. In brief, the first antibody was a polyclonal rabbit anti-human α_1 -Pi immune serum (Sigma, Poole, Dorset, UK) and the second antibody was a sheep anti-rabbit α_1 -Pi conjugated to peroxidase (The Binding Site, Birmingham, UK). The reaction was quantified with the chromogenic substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (Sigma) and the absorbance read at 490 nm. α_1 -Pi purified from human plasma and of a known concentration was used as a standard.

The concentrations of SLPI and elafin were measured using established enzyme-linked immunosorbent assays (ELISA) as described previously [19]. In brief, anti-elafin and anti-SLPI polyclonal rabbit immunoglobulin G (IgG) were obtained by protein A chromatography [19]. The secondary antibody was a goat horseradish peroxidase-conjugated anti-rabbit IgG adsorbed with human serum proteins (Sigma). The reaction was developed as described above for α_1 -Pi. Recombinant human elafin, a gift from J. Fitton (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK), and recombinant SLPI, a gift from R. Heinzl-Wieland (Grunenthal, Stolberg, Germany), were used as standards.

Measurement of albumin

The albumin level in BAL was determined by immunonephelometry (Kallestad QM300 nephelometer, Montreal, Quebec, Canada), using anti-human albumin antibodies (Sanofi Diagnostics Pasteur, Montreal, Canada).

Total neutrophil elastase inhibitory activity

The neutrophil elastase inhibitory activity (EIA) in BAL was assessed as described previously [15]. In brief, 100 μ L BAL supernatant was added to 10 μ L (300 ng) of active-site titrated human HNE (Elastin Products Company, Owensville, MO, USA) and incubated at 37°C for 15 min. The control consisted of HNE incubated with 100 μ L saline. The mixtures were incubated with the chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeO-SAAPVNA) (Sigma; final concentration 0.5 nM). After a further 5 min to allow equilibration, hydrolysis of the substrate was recorded at 410 nm over a 5-min period. The amount of BAL fluid sample required to inhibit HNE completely was determined by extrapolation of the linear part of the inhibitory curve to the abscissa, assuming a 1:1 ratio of HNE to inhibitors known to reside in BAL (elafin, SLPI and α_1 -Pi). With this assumption (these three inhibitors have been demonstrated to form a 1:1 stoichiometric complex with HNE [10]), it is possible to calculate EIA, expressed in nM. This value is independent of the affinities (K_i) of the different inhibitors with HNE [20].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot analysis

Twenty microlitres of BAL supernatant were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [15]. Western blotting was performed using Immobilon-NC transfer membranes (Millipore, Bedford, MA, USA). Blocking of nonspecific binding sites was performed overnight at room temperature in phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 5% skimmed milk powder. Membranes were then incubated with rabbit anti-elafin or anti-SLPI IgG at a dilution of 1:1,000 in blocking buffer, for 1 h at room temperature. After a further 20-min incubation with goat anti-rabbit IgG conjugated to peroxidase (1:2,500 dilution; Sigma), the blots were developed by enhanced chemiluminescence, according to the manufacturer's instructions (Dupont, NEN Research Products, Boston, MA, USA). The blots were exposed to Reflection NEF autoradiography film (Dupont).

Statistics

Inhibitor concentrations and activities from the different groups of subjects are expressed as mean \pm SEM and were analysed using a Kruskal–Wallis (unpaired) test followed by a nonparametric Fisher-like test. Linear correlations were used to measure the degree of association between variables. In all cases, $p < 0.05$ was considered as significant.

Results

Bronchoalveolar lavage cell differentials

There was no significant difference in BAL cell differentials between patients at risk of ARDS who subsequently developed the disease and those who did not. Therefore, values from at-risk patients were pooled and included in a single group (table 3). The mean total cell

Table 3. – Bronchoalveolar lavage cell differentials in collected samples from at-risk and established acute respiratory distress syndrome patients

	N	M	L	E
At-risk	4.0 (0–7)	84 (66–94)	12.0 (2–19)	0 (0–2)
Established disease	48 (11–79)	41 (23–78)	11 (9–18)	0 (0–1)

Results are expressed as median percentages, with ranges in parentheses. Patients with established ARDS had a very significantly increased percentage of neutrophils compared with patients at risk of ARDS ($p=0.0001$). N: neutrophils; M: macrophages/monocytes; L: lymphocytes; E: eosinophils.

count in BAL from patients at risk of ARDS was 7.8×10^6 ($\pm 2.3 \times 10^6$) and 8.6×10^6 ($\pm 2.1 \times 10^6$) from patients with established disease ($p=0.47$). Patients with established ARDS had a highly significantly increased percentage of neutrophils compared with at-risk patients ($p=0.0001$, table 3).

Levels of antiproteases and albumin in bronchoalveolar lavage fluid

There was no statistically significant difference in levels of albumin between normal subjects ($0.23 \pm 0.09 \mu\text{M}$) and at-risk patients who did not develop ARDS ($2.23 \pm 4.84 \mu\text{M}$) ($p=0.56$), although there was a trend towards significance between normal subjects and at-risk patients who did develop ARDS ($2.68 \pm 3.21 \mu\text{M}$) ($p=0.083$) and between normal subjects and patients with established ARDS ($4.06 \pm 6.27 \mu\text{M}$) ($p=0.065$). This may suggest increased alveolar-capillary permeability in patients progressing to ARDS and in those with established ARDS.

Figure 1 shows that all BAL fluid from patients had significantly increased levels of elafin, SLPI and α_1 -Pi, compared with normal subjects. When BAL fluid from at-risk patients was examined, BAL from those patients who went on to develop ARDS had higher amounts of SLPI than that from patients who did not ($p=0.0083$; fig. 1a). In contrast, elafin and α_1 -Pi levels were not significantly different ($p=0.38$ and 0.54 , respectively). When BAL fluid from at-risk patients was compared with BAL fluid from patients with established ARDS, SLPI levels were found to be increased in the latter ($p=0.0003$; fig. 1a). Similarly, α_1 -Pi levels were significantly increased in BAL from patients with established ARDS compared with at-risk patients who did not develop ARDS ($p=0.0089$; fig. 1c). In contrast, elafin levels did not change significantly ($p=0.08$; fig. 1b).

Figure 2 shows that the total antigenic level of elastase inhibitors (SLPI, elafin, and α_1 -Pi) was significantly increased in BAL fluid from patient groups than in healthy individuals. Levels were also significantly increased in the samples from patients with established ARDS, compared to the samples from at-risk patients but who did not progress to ARDS ($p=0.0033$).

Elastase inhibitory activity in bronchoalveolar lavage

The EIA was measured in BAL fluid to assess whether the increase in antigenic levels of elastase inhibitors (EI)

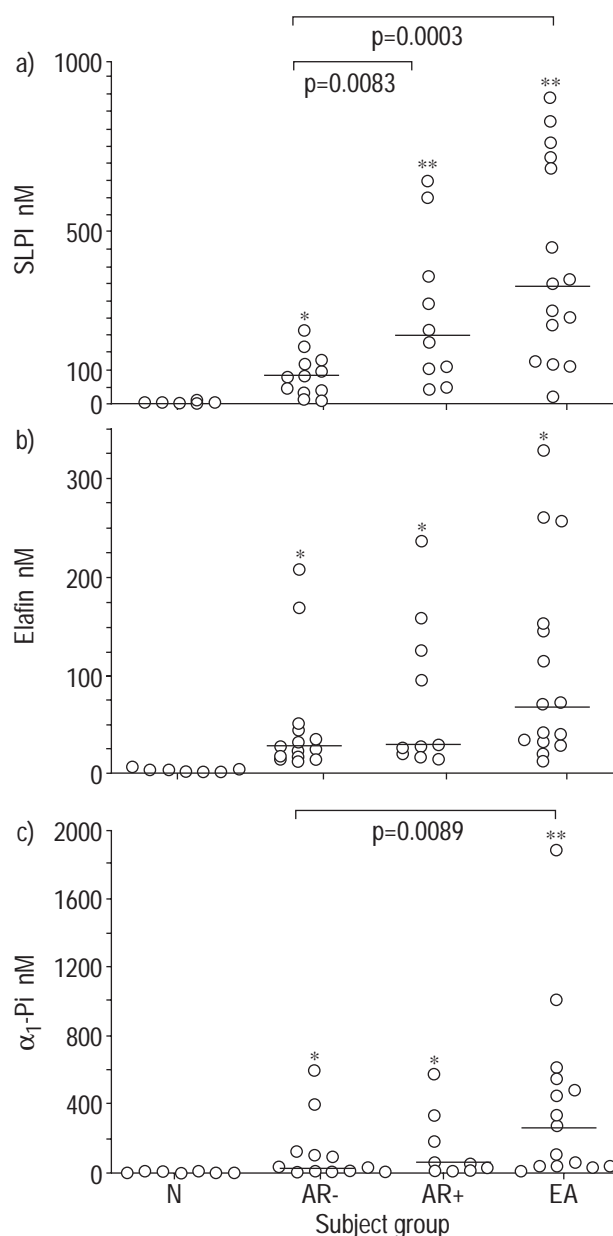


Fig. 1. – Levels of a) secretory leukocyte proteinase inhibitor (SLPI), b) elafin and c) alpha-1-proteinase inhibitor (α_1 -Pi) in bronchoalveolar lavage (BAL) fluid. Individual values of elafin, SLPI and α_1 -Pi levels are shown for the four groups of subjects. N: normal subjects; AR-: at-risk patients who did not develop acute respiratory distress syndrome (ARDS) at a later stage; AR+: at-risk patients who did develop ARDS at a later stage; EA: patients with established ARDS. Levels were assessed by enzyme-linked immunosorbent assay. *: Significantly different from normal subjects ($p<0.05$); **: significantly different from normal subjects and at-risk patients who did not develop ARDS. The median is represented by a horizontal bar.

corresponded with increased activity. In keeping with the ELISA data, all patient groups had increased EIA, compared with the group of normal subjects (fig. 3a). In most of the subjects, the EIA/ α_1 -Pi ratio was >1 , suggesting that α_1 -Pi was not the only antiprotease active in BAL (fig. 3b).

When compared internally, BAL from the three different groups of patients did not show any significant difference in terms of EIA (fig. 3a), which suggests that in ARDS a

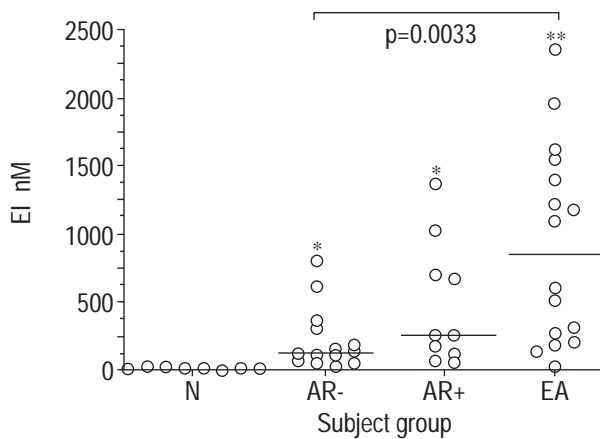


Fig. 2. – Total elastase inhibitor (elafin, secretory leukocyte proteinase inhibitor, and alpha-1-proteinase inhibitor) concentrations (EI) (derived from values presented in fig. 1) in bronchoalveolar lavage fluid from the four groups of subjects: N: normal subjects; AR-: at-risk patients who did not develop acute respiratory distress syndrome (ARDS) at a later stage; AR+: at-risk patients who did develop ARDS at a later stage; EA: patients with established ARDS. Levels were assessed by enzyme-linked immunosorbent assay. *: Significantly different from normal subjects ($p < 0.05$); **: significantly different from normal subjects and at-risk patients. The median is represented by a horizontal bar.

proportion of the total inhibitors, in particular SLPI and α_1 -Pi (antigen levels of which were significantly increased in BAL fluid from established ARDS patients; figs. 1 and 2), was inactive. In keeping with this, seven out of 16 subjects with established ARDS exhibited an EIA/ α_1 Pi ratio < 1 (fig. 3b), suggesting that a considerable proportion of inhibitors was inactive. Indeed, EIA/ α_1 -Pi ratios in BAL fluid from patients with established ARDS were significantly lower than in at-risk patients who did not develop ARDS ($p = 0.0012$). Furthermore, when EIA was corrected for total EI, there was a trend towards a reduction in the values going from normal subjects through to patients with ARDS. The difference became statistically significant when comparing the established ARDS group with normal subjects (fig. 3c, $p = 0.045$).

Western blot analysis

When BAL from ARDS patients was examined by SDS-PAGE followed by Western blotting (fig. 4) to assess the biochemical characteristics of the three inhibitors studied (α_1 -Pi, SLPI and elafin), it was found that α_1 -Pi migrated at the expected location (52 kDa, fig. 4). Similarly, most of the SLPI-immunoreactive species migrated at the expected position (12 kDa), although some material was clearly detectable at a higher position (55 kDa). In contrast, virtually all of the immunoreactive elafin found in ARDS BAL migrated at a higher position (50 kDa) than did recombinant elafin (10 kDa).

Discussion

Within the lung, an imbalance in favour of excess proteinase has been implicated in such diverse diseases as emphysema, bronchiectasis, cystic fibrosis and ARDS. However, apart from the hereditary form of emphysema in which a link has been established between disease de-

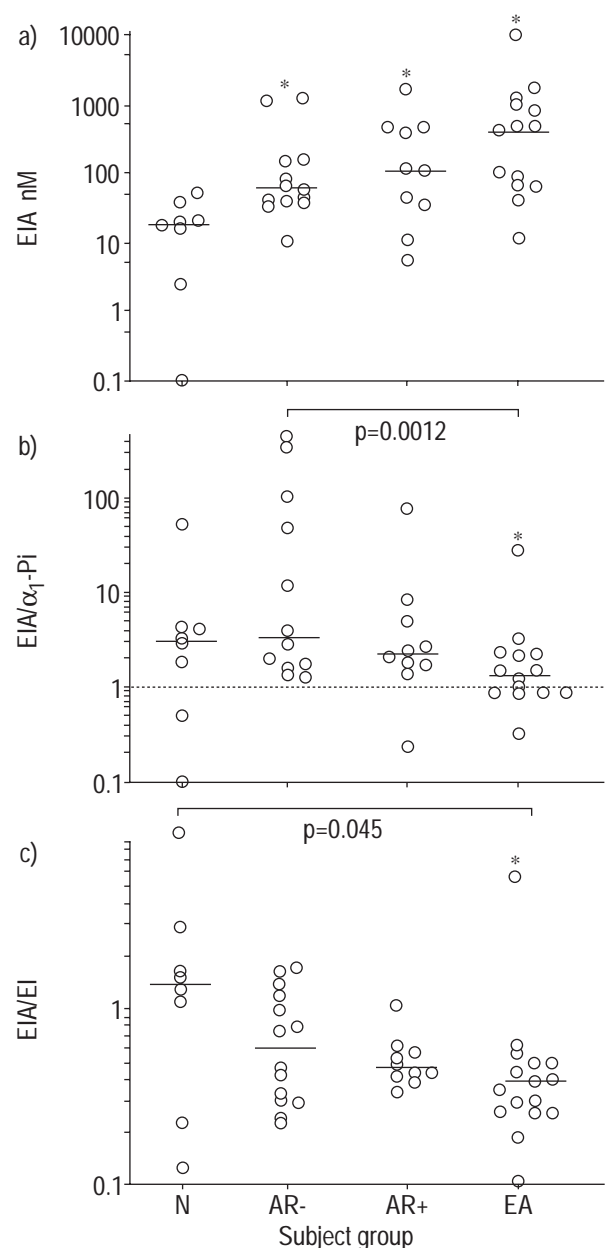


Fig. 3. – Elastase inhibitory activity (EIA) in bronchoalveolar lavage (BAL) from the four groups of subjects: N: normal subjects; AR-: at-risk patients who did not develop acute respiratory distress syndrome (ARDS) at a later stage; AR+: at-risk patients who did develop ARDS at a later stage; EA: patients with established ARDS. One-hundred microlitres of BAL was added to 10 μ L (300 ng) of human neutrophil elastase (HNE). Residual HNE activity was measured with MeO-SAAPVNA (see *Materials and methods*). EIA was also expressed relative to alpha-1-proteinase inhibitor (α_1 -Pi) and relative to total elastase inhibitors (EI). *: Significantly different from normal subjects ($p < 0.05$). The median is represented by a horizontal bar.

velopment and α_1 -Pi deficiency [7], no conclusive evidence has been produced implicating proteases in the pathogenesis of other inflammatory disease states such as ARDS. In addition, α_1 -Pi is not the only elastase inhibitor present in the lung, and low molecular weight elastase inhibitors such as SLPI and elafin are increasingly thought to be important as part of the "antiproteinase shield" in lung defences.

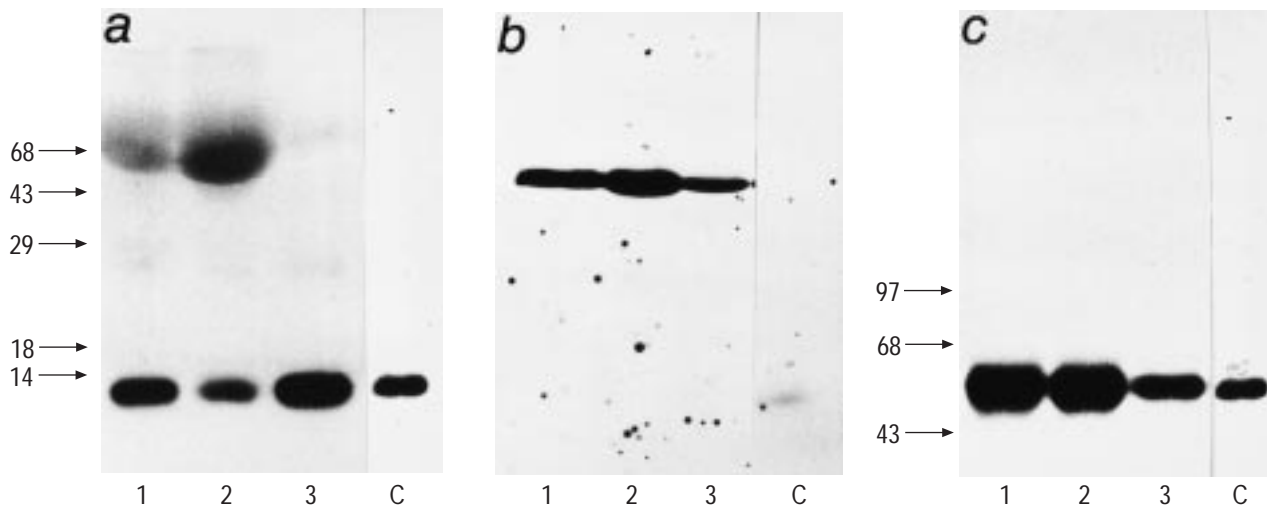


Fig. 4. – Analysis of a) secretory leukocyte proteinase inhibitor (SLPI), b) elafin and c) alpha-1-proteinase inhibitor (α_1 -Pi) in bronchoalveolar lavage (BAL) fluid samples. Twenty microlitres of BAL samples were loaded and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis followed by Western blot analysis. Lanes 1–3: BAL fluid from three patients with established acute respiratory distress syndrome; lane 4: recombinant SLPI, elafin and purified α_1 -Pi were used as controls. Migration is expressed in kilodaltons, using prestained low molecular weight markers (Gibco-BRL) as standards.

Prior to the present study, others have compared BAL from patients with established ARDS and BAL from normal individuals. These studies have suggested that levels of α_1 -Pi [6, 21, 22] and SLPI [21] were increased in established ARDS.

To the authors' knowledge, the present study is the first to assess the intra-alveolar levels of the three major antiproteases known to inhibit HNE (α_1 -Pi, SLPI, and elafin), not only in established ARDS, but also at earlier time points in patients at a significant risk of developing the disease.

The main findings of the present study are: 1) the levels of the major antiproteases were increased not only in established ARDS but also in the at-risk period; 2) the increased levels in the at-risk period provide further evidence for enhanced early inflammation prior to overt clinical disease [3, 20]; and 3) significantly raised levels of SLPI (but not elafin or α_1 -Pi) were found in the alveolar airspaces of at-risk patients who subsequently progressed to ARDS than in those who did not.

Interestingly, it was also found that SLPI levels were highest in patients with established ARDS, in keeping with earlier work by IDELL *et al.* [21]. This observation held true when SLPI levels were corrected for the number of neutrophils in each patient. However, the state of activation of intra-alveolar neutrophils might be important: activated neutrophils could also be producing SLPI, both in at-risk patients and in patients with established ARDS, consistent with findings *in vitro* [23].

α_1 -Pi levels were significantly elevated in BAL from patients with established ARDS than in BAL fluid from at-risk patients. Although α_1 -Pi can be secreted locally by inflammatory and structural cells [24, 25], most of the α_1 -Pi reaching the pulmonary airspace is thought to be derived from the circulation. This point was reinforced by the finding that all of the α_1 -Pi recovered in BAL had the same molecular weight as that produced in the liver, which has a different glycosylation pattern and molecular weight from epithelial cell-derived α_1 -Pi [24, 25] (fig. 4).

Alpha-2-macroglobulin (α_2 -M) is a further elastase inhibitor derived from the circulation (not studied here) which has been found in established ARDS [22]. α_2 -M–HNE complexes have residual enzymatic activity against low molecular weight substrates such as MeO-SAAPV-NA, which explains why certain studies have found free neutrophil elastase activity in ARDS BAL [22]. However, α_2 -M is present at very low concentrations in the BAL and represents <1% of the α_1 -Pi molar concentration [22].

When total EI (SLPI, elafin and, α_1 -Pi) were examined, they were found to be significantly increased (compared with normal controls) both in the at-risk period and in established disease. When patient samples were compared internally, levels in the established ARDS group were significantly increased, compared with the at-risk group, reflecting the increase in SLPI and α_1 -Pi in these samples (fig. 2). The present results showing enhanced EIA in all patient samples, compared with normal subjects, confirms previously published work by MAUNDER *et al.* [26] and WEWERS *et al.* [22]. In most samples, the EIA/ α_1 -Pi ratio was >1 (fig. 3), clearly showing that α_1 -Pi is not the only antiprotease active in BAL. Significantly, the ratio was >1 in normal subjects (six out of eight individuals) and in at-risk patients (14 out of 14 patients not developing ARDS and nine out of 10 patients developing ARDS) but was often <1 in the established ARDS group (nine out of 16 patients), showing that in the latter group, a number of patients had inactivated α_1 -Pi. When EIA was expressed relative to total inhibitors (EI), the ratio EIA/EI was >1 in 75% of the normal subjects (suggesting the existence of an unidentified elastase inhibitor) and was higher than in patients with established ARDS (fig. 3). However, it was not significantly different between the groups of patients (although there was an obvious trend towards the reduction of the ratio with progression of the disease) (fig. 3). This would suggest that a proportion of these inhibitors are inactivated in ARDS, when the inflammatory process is at an advanced stage, and that ARDS is associated with increased

inactivation of antiproteases in the alveolar airspace. There are several potential *in vivo* mechanisms for this observation. Firstly, antiprotease inactivation could result as a consequence of complex formation with HNE. With regard to α_1 -Pi, Western blot analysis on the collected BAL samples failed to reveal significant quantities of the typical 80 kDa band representing the α_1 -Pi-HNE complex. With regard to SLPI and elafin, complexes with HNE cannot be ruled out since they are not resistant to SDS [27, unpublished data]. A second possibility for antiprotease inactivation is cleavage at or near the active site. For example, in cystic fibrosis, α_1 -Pi is cleaved at its active site, generating a 49 kDa inactive protein, migrating more quickly than the native 52 kDa form [28]. Similarly, SLPI can be proteolytically degraded both *in vitro* and *in vivo* [23, 29]. In the present study, it was shown that none of the three inhibitors was cleaved to any significant degree (fig. 4). In contrast elafin and, to a lesser degree, SLPI are complexed to unknown molecules. The complexes have a molecular weight close to 50 kDa, similar to one of the complexes found in BAL from farmer's lung disease [15]. Since α_1 -Pi cannot account for all of the EIA (see above), and assuming that α_1 -Pi, SLPI and elafin are the only elastase inhibitors in most BAL samples, these data suggest that SLPI and X-elafin complexes are significantly active in these samples. This potentially represents an important observation, as an active X-elafin complex in the interstitium would provide local protection against neutrophil elastase during inflammatory processes, regulating proteinase activity precisely where it is most needed within the target organ.

The overall increase in proteinase inhibitors, and in particular SLPI, in at-risk patients is not associated with protection from the development of overwhelming inflammatory lung injury and ARDS. One explanation for this could be that antiproteases are released as an aborted and unsuccessful attempt to control HNE-driven injury within the inflammatory response.

In conclusion, the present study highlights the importance of secretory leukocyte proteinase inhibitor and other antiproteases, not only in patients with established acute respiratory distress syndrome, but also in the early "at-risk" period prior to the development of clinically apparent lung injury, at a time when an enhanced intrapulmonary inflammatory response is underway. This is in agreement with *in vitro* data showing that some of these antiproteases are under the control of pro-inflammatory cytokines in alveolar cells [19]. The dissection of the regulation of secretory leukocyte proteinase inhibitor and other antiproteases (such as the tissue inhibitor for metalloproteinase, which has also been found to be increased in acute respiratory distress syndrome) [30, 31] secreted at this early time point may offer therapeutic potential in attenuating the pulmonary inflammation in early acute respiratory distress syndrome and reducing morbidity and mortality.

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