

## MISCELLANEOUS

# Relationship of anti-GM-CSF antibody concentration, surfactant protein A and B levels, and serum LDH to pulmonary parameters and response to GM-CSF therapy in patients with idiopathic alveolar proteinosis

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**Background:** Conventional measures of the severity of alveolar proteinosis (AP) include alveolar-arterial oxygen gradient ( $[A - a]D_{O_2}$ ), vital capacity (VC), and carbon monoxide transfer factor ( $T_{lco}$ ), but alternative serological measures have been sought. Granulocyte-macrophage colony stimulating factor (GM-CSF) neutralising autoantibody is found in patients with idiopathic acquired AP. We have investigated the interrelationships between the levels of this antibody and those of surfactant protein (SP)-A and -B, lactate dehydrogenase (LDH), and conventional measures of disease severity, and the capacity of these parameters to predict the response to rhGM-CSF treatment.

**Methods:** Blood levels of anti-GM-CSF antibodies, SP-A, SP-B, LDH, and  $[A - a]D_{O_2}$ , VC, and  $T_{lco}$  were measured before rhGM-CSF treatment and every 2 weeks thereafter in 14 patients with AP.

**Results:** At baseline, high levels of anti-GM-CSF antibodies and increased SP-A and SP-B levels were seen in all patients, and LDH was raised in 83%. SP-A was highly correlated with  $[A - a]D_{O_2}$ , VC, and  $T_{lco}$  ( $p \leq 0.02$ ), but other markers were not. Only a normal LDH level was predictive of a response to rhGM-CSF treatment ( $p = 0.03$ ). During treatment a correlation between conventional and serological variables within patients was seen only between SP-A and  $[A - a]D_{O_2}$  ( $p = 0.054$ ), LDH levels and  $[A - a]D_{O_2}$  ( $p = 0.010$ ), and LDH levels and VC ( $p = 0.019$ ).

**Conclusions:** Of the serological parameters studied, only SP-A and LDH levels were correlated with conventional measures of disease severity, with LDH most accurately reflecting  $[A - a]D_{O_2}$  and vital capacity. Only a normal LDH level predicted a higher likelihood of response to treatment with GM-CSF.

Alveolar proteinosis (AP) is an uncommon lung disorder characterised by excessive surfactant accumulation within the alveoli.<sup>1–3</sup> There are three distinct forms of AP. The rare congenital form is transmitted in an autosomal recessive manner, most often due to homozygosity for a frameshift mutation in the surfactant protein-B (SP-B) gene.<sup>4</sup> AP also rarely complicates an underlying malignancy, usually of haematopoietic origin.<sup>5</sup> However, more than 90% of AP cases are a primary acquired disorder without familial predisposition, and have been considered to be of "idiopathic" aetiology.<sup>1–3</sup>

It was shown 20 years ago that the bronchoalveolar lavage (BAL) material and serum from patients with AP had "immunoinhibitory" activity.<sup>6</sup> We have subsequently identified the immunoinhibitory substance as an IgG autoantibody which neutralises the biological activity of granulocyte-macrophage colony stimulating factor (GM-CSF).<sup>7–9</sup> Endogenous GM-CSF is required for normal surfactant phospholipid and protein clearance by alveolar macrophages.<sup>10</sup>

A significant issue in the management of patients with idiopathic AP remains the difficulty in accurately and easily assessing disease severity. The most widely accepted measure of disease severity is the alveolar-arterial oxygen gradient ( $[A - a]D_{O_2}$ )<sup>2,11</sup> which requires arterial blood gas sampling. The spirometric parameters of vital capacity (VC) and lung carbon monoxide transfer factor ( $T_{lco}$ )<sup>2,12</sup> and functional assessments of exercise capacity using either walk tests or formal ergometry are also considered useful measures of disease severity<sup>13,14</sup> but are not universally available, are time consuming, or expensive. A robust, inexpensive, minimally invasive and readily available means of disease assessment in patients with AP would therefore be useful.

The accumulated surfactant material from patients with AP has been shown to contain large amounts of phospholipid,<sup>15</sup> SP-A,<sup>16–19</sup> SP-B,<sup>17</sup> and SP-D.<sup>20,21</sup> These surfactant-associated proteins are also detectable in the blood of many patients.<sup>18,20,22</sup> However, while levels are often considerably raised in patients with AP, moderate increases may also be seen in patients with a range of other disorders including idiopathic pulmonary fibrosis, sarcoidosis, pneumonia, respiratory failure, and ARDS, diminishing the diagnostic specificity of these assays.<sup>18,20,23–25</sup> Despite their suboptimal diagnostic specificity, the serum levels of these proteins may reflect their alveolar levels, making them potentially useful in disease monitoring. Similarly, serum levels of lactate dehydrogenase (LDH) are raised in patients with AP,<sup>12,26,27</sup> may correlate with the  $[A - a]D_{O_2}$ , and decline following effective treatment.<sup>27</sup>

We have developed a method to measure accurately the concentration of anti-GM-CSF antibody in serum and report the relationship between levels of anti-GM-CSF antibody, SP-A, SP-B, and LDH and conventional measures of disease severity in patients with idiopathic AP. Our group and others have recently shown that the subcutaneous administration of recombinant human GM-CSF (rhGM-CSF) is effective in 40–50% of patients with AP,<sup>22,28–30</sup> and we also explore the ability of these parameters to predict a response to such treatment.

## METHODS

### Patients and spirometric tests

The samples analysed were obtained from patients participating in a prospective clinical trial of subcutaneous rhGM-CSF which was open to accrual from August 1995 to September 1998. The

details of the study have been reported elsewhere.<sup>22</sup> Briefly, eligibility required a diagnosis of acquired AP confirmed by central pathological review, symptomatic disease, and the absence of active pulmonary infection. The study was conducted in accordance with the Declaration of Helsinki, approved by the institutional ethics committee at participating institutions, and all patients provided written informed consent.

Serum levels of LDH, SP-A and SP-B, arterial blood gas measurements, spirometric tests with  $T_{LCO}$ , and the concentration of anti-GM-CSF antibodies were determined before treatment and every 2 weeks during treatment with rhGM-CSF. Spirometric testing with  $T_{LCO}$  was performed according to institutional protocols<sup>31,32</sup> and all results were expressed as a percentage of predicted values using individual institutional reference ranges.

#### rhGM-CSF administration

Patients treated in Australasia and Europe (n=11) received bacterially synthesised rhGM-CSF (Leucomax, Schering-Plough; specific activity  $0.66\text{--}1.66 \times 10^7$  IU/mg protein,  $\leq 25$  Eu endotoxin/vial by the *Limulus amoebocyte* assay; data on file, Schering-Plough, Australia) and patients treated in the United States (n=4) received yeast derived rhGM-CSF (Leukine, Immunex, WA; specific activity  $5.6 \times 10^6$  IU/mg protein,  $\leq 25$  Eu endotoxin/vial; data on file, Immunex, WA). As described elsewhere,<sup>22</sup> rhGM-CSF was started at  $3.0 \mu\text{g}/\text{kg}$  subcutaneously daily for 5 days and increased to  $5.0 \mu\text{g}/\text{kg}$  from day 6 onwards for a minimum of 6 weeks in the absence of prohibitive toxicity. Dose escalation of rhGM-CSF to 7.5, 10, 15, 20 and  $30 \mu\text{g}/\text{kg}$  was allowed in patients who did not improve with  $5 \mu\text{g}/\text{kg}/\text{day}$  and did not display a  $\geq 1.5$ -fold increase in neutrophil count with this dose.<sup>33</sup>

#### Response criteria

As defined previously,<sup>22</sup> a complete response required normalisation of the CT scan, spirometric parameters,  $T_{LCO}$  (using institutionally specified normal ranges), and arterial oxygenation (defined by the age appropriate calculated  $[A - a]Do_2$  on room air  $\pm 2$  SD).<sup>34</sup> A partial response was defined as an improvement of  $\geq 50\%$  in one or more of the following parameters during the study: (1) radiographically defined volume of pulmonary abnormality, (2)  $T_{LCO}$ , or (3)  $[A - a]Do_2$ .

#### Measurement of anti-GM-CSF IgG

Anti-GM-CSF antibodies were assayed centrally in a blinded manner using serum samples that had been stored at  $-70^\circ\text{C}$  until analysis. Anti-GM-CSF antibody was purified from a patient and used as a standard.<sup>30</sup> Briefly, the antibody was precipitated by 33% ammonium sulfate sedimentation before being affinity purified using NHS activated HiTrap (Amersham Pharmacia Biotech UK Ltd) coupled with rhGM-CSF (*Escherichia coli* derived; Kirin Brewery Co Ltd, Gunma, Japan) according to the manufacturer's instructions. The purity of the isolated antibody was confirmed by SDS gel electrophoresis and its concentration determined by both a protein assay (Bio Rad Co Ltd, CA, USA) and a sandwich-type enzyme linked immunosorbent assay (ELISA) with non-labelled and peroxidase labelled anti-human IgG antibodies (Dako Corporation, Carpinteria, CA, USA) using human IgG (Sigma-Aldrich) as a standard for quantitative analysis. The purity of the isolated antibody, as determined by IgG concentration per total protein concentration, was more than 0.95.

Anti-GM-CSF antibody concentrations were measured in test samples in a similar manner to that previously described.<sup>8</sup> Briefly, serum samples were diluted 1:1500 with phosphate buffered saline (PBS) containing 0.1% goat serum and 0.1% Tween 20. Separate  $50 \mu\text{l}$  aliquots of diluted serum and the purified anti-GM-CSF antibody standard were incubated at room temperature for 40 minutes in ELISA plates previously coated overnight at  $4^\circ\text{C}$  with  $1 \mu\text{g}/\text{ml}$  rhGM-CSF (Kirin Brewery Co Ltd) and blocked for 1 hour with 1% bovine serum

albumin/PBS. After washing four times with PBS 0.1% Tween 20 (PBST),  $50 \mu\text{l}$  of 10 mM ammonium acetate buffer (pH 6.0) was added to each well and the plates were incubated at room temperature for a further 15 minutes. The plates were washed with PBS. Antibody captured by rhGM-CSF were detected by peroxidase labelled anti-human IgG F(ab)<sub>2</sub> antibody (Dako). Tetramethylbenzidine was used as a substrate and absorbance was measured at 450 nm. All assays were performed in duplicate and the mean of the two results used.

#### SP-A and -SP-B assays

SP-A and SP-B were measured as previously described.<sup>17,23,24</sup> The antigens were measured by ELISA inhibition assays using antibodies raised against alveolar proteinosis derived SP-A and mature SP-B. Briefly, in order to free the SP-A and SP-B from any associated plasma or surfactant components, aliquots were first treated with EDTA, SDS, and Triton X-100. Serial dilutions of the samples in PBST containing 0.25% BSA (w/v) were incubated in an ELISA plate with aliquots of the respective antibody. Free antibody was captured using a second ELISA plate coated with purified SP-A or SP-B ( $1 \mu\text{g}/\text{ml}$ ) and the amount measured using alkaline phosphatase conjugated IgG against rabbit immunoglobulins and 15 mM disodium *p*-nitrophenyl phosphate in 1.0 M diethanolamine and 0.5 mM  $MgCl_2$  as a substrate. After 1 hour the absorbance of the substrate was measured at 405 nm using a Dynatech MR5000 reader (Dynatech Laboratories, Chantilly, VA, USA). The AssayZap program (Biosoft, Ferguson, MO, USA) was used to generate a standard curve and to compute the concentration of SP-A and SP-B in each sample. All samples were assayed in duplicate at four serial dilutions. Standards, assayed in quadruplicate, were included in each ELISA plate at eight serial dilutions (range 7.8–1000 ng/ml for SP-B and 1.95–250 ng/ml for SP-A, both  $r > 0.99$ ). The antibodies used do not react with any other known antigens and the assays have coefficients of variance of  $\sim 6\%$ .

#### Serum LDH

LDH assays were performed at accredited diagnostic laboratories in each participating institution using standard automated analysers. As the normal range for LDH varies, results were standardised by expressing them as a proportion of the upper limit of the normal range (ULN) for that institution.

#### Statistical methods

The normality of the distribution of each variable was assessed using the Kolmogorov-Smirnov test and variables that failed this test were subjected to logarithmic transformation. Comparisons between groups were performed using the Fisher exact test for categorical data and the Mann-Whitney test for continuous data. Changes in variables with treatment were analysed with paired *t* tests or Wilcoxon paired two sample tests. For any assay results to be considered "simultaneous" for correlation analyses, the studies must have been performed within 24 hours. Correlation of variables between patients was assessed using the Spearman rank correlation coefficient. Linear regression lines and their 95% confidence intervals were calculated using the least squares method. Correlation of variables within patients was assessed using a modified signed rank test (see Appendix). All *p* values are two sided, with  $p < 0.05$  being regarded as significant. No formal adjustment for multiple comparisons was made. Analyses were performed using Minitab 9.2 (Minitab Inc, State College, PA, USA) or GraphPad Prism 3.02 (GraphPad Software Inc, San Diego, CA, USA).

## RESULTS

### Pretreatment characteristics

As previously reported,<sup>22</sup> 14 eligible patients were enrolled in the study, although pretreatment serum samples were only

**Table 1** Baseline characteristics of patients (n=12) with alveolar proteinosis evaluable

Characteristic	Median (range)
Age (years)	31.5 (14–45)
M/F	8/4
No (%) life long non-smoking patients	6 (50%)
Months from diagnosis	28 (1–96)
No (%) with prior therapeutic whole lung lavage	9 (75%)
No of prior therapeutic lavages	8 (2–16)
Months since last therapeutic lavage	6 (1.5–19)
Arterial PO <sub>2</sub> (mm Hg)	63.5 (51.1–83.5)
[A–a]DO <sub>2</sub> (mm Hg)	45.5 (18.2–52.2)
Vital capacity (% predicted)	65 (33–104)
TlCO (% predicted)	45 (19–78)
No (%) with raised serum LDH	10 (83%)
Serum SP-A (µg/ml)*	1.53 (0.57–6.02)
Serum SP-B (µg/ml)†	18.7 (8.5–48.9)
Anti-GM-CSF antibody (µg/ml)	244.7 (50.7–617.9)

PO<sub>2</sub>=arterial oxygen tension; [A–a]DO<sub>2</sub>=alveolar-arterial oxygen gradient; TlCO=carbon monoxide transfer factor; SP=surfactant protein; GM-CSF=granulocyte-macrophage colony stimulating factor.  
\*Normal range for serum level of SP-A is <0.25 µg/ml.  
†Normal range for serum level of SP-B is <2.6 µg/ml.

available from 12. The concentrations of anti-GM-CSF antibody were normally distributed (mean (SD) 256.7 (166.5) µg/ml, table 1). While SP-B levels were lower in patients diagnosed closer to enrolment to the study (less than the median of 28 months before entry: 14.8 µg/ml v 33.0 µg/ml; p=0.007), no other significant differences in SP-A, SP-B, or anti-GM-CSF antibody levels were evident (table 2).

Baseline levels of SP-A were strongly correlated with [A – a]DO<sub>2</sub>, VC (% predicted), and TlCO (% predicted) and inversely with arterial PO<sub>2</sub> (table 3, figs 1 and 2), but the baseline concentrations of anti-GM-CSF antibody, SP-B, and LDH levels showed no significant correlation with these measures of disease severity.

### Associations with response to GM-CSF treatment

#### Anti-GM-CSF antibody levels

As previously reported,<sup>22</sup> five of the 14 patients responded to treatment with the initial dose of 5 µg/kg/day. Two of 11 patients with a raised serum LDH level responded to rhGM-CSF treatment (18%) compared with all three patients with normal levels (100%, p=0.03). The level of pretreatment anti-GM-CSF antibody did not differ according to therapeutic

response (responders: median 241 µg/ml (range 85–410); non-responders: median 230 µg/ml (range 51–618), p=0.9).

The anti-GM-CSF antibody concentration fell in all patients during their initial treatment with 5 µg/kg/day rhGM-CSF by a median of 75 µg/ml (range –215 to –17); p=0.0007. However, the change in antibody concentration (Δ) did not differ between responders (median Δ=–90 µg/ml; range –215 to –17) and non-responders (median Δ=–60 µg/ml; range –93 to –17), p=0.4. A similar pattern was seen in the four evaluable patients who underwent either dose escalation (from 7.5 to 20 µg/kg/day GM-CSF) or re-treatment after a prior response to rhGM-CSF; median Δ=–95 µg/ml (range –150 to –42), p=0.03.

Following cessation of rhGM-CSF treatment, sequential assessment revealed no clear pattern of anti-GM-CSF antibody concentration among the three patients with available data (median Δ=–6.5 µg/ml; range –25.4 to +63). Anti-GM-CSF antibody concentrations (µg/ml) from individual patients at the completion of GM-CSF treatment compared with last follow up (time after last dose of GM-CSF) were 81.6 v 56.2, 62.2 v 55.7, and 139 v 202 at 321, 118 and 57 days, respectively.

#### LDH, SP-A and SP-B

As previously reported,<sup>22</sup> there were no differences in pretreatment levels of LDH, SP-A, or SP-B between responders and non-responders. Also, the levels did not change significantly with treatment, either for the group as a whole or when subdivided into responders and non-responders (data not shown).

### Correlations between serological markers and conventional measures of disease severity

Relationships between the conventional measures of disease severity ([A – a]DO<sub>2</sub>, TlCO (% predicted), and VC (% predicted)) and each of the serological markers under investigation (anti-GM-CSF antibody levels, SP-A, SP-B, and LDH) were investigated individually for each of the 14 studied patients and for each pair of variables. Within patients, SP-A levels correlated with [A – a]DO<sub>2</sub> (p=0.054) and LDH levels correlated with [A – a]DO<sub>2</sub> (p=0.010) and VC (% predicted) (p=0.019), whereas levels of anti-GM-CSF antibody and SP-B did not show a correlation with any of the conventional variables (table 4).

Among the serum markers, significant cross correlation was seen only between SP-B and LDH (p=0.027, data not shown). Levels of anti-GM-CSF antibody showed no relationship with any of the other serum parameters measured.

**Table 2** Pretreatment anti-GM-CSF antibody concentrations and surfactant protein (SP) levels according to patient and disease features

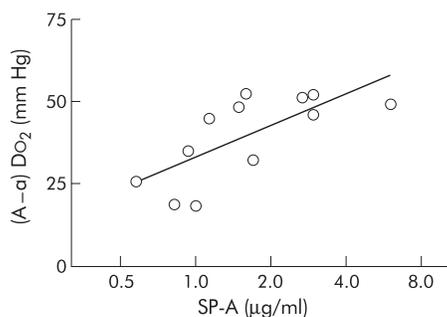
Feature	Anti-GM-CSF Ab (µg/ml)	p value	SP-A (µg/ml)	p value	SP-B (µg/ml)	p value
Age (years)						
<32 (n=6)	208 (51–618)	NS	2.8 (0.9–6.0)	NS	16.5 (8.5–37.5)	NS
≥32 (n=6)	320 (85–410)		1.3 (0.6–1.7)		24.6 (9.3–48.9)	
Sex						
Male (n=9)	245 (74–410)	NS	1.1 (0.6–29.6)	0.06	17.1 (9.3–37.5)	NS
Female n=3	216 (51–618)		2.8 (1.5–6.0)		20.2 (8.5–48.9)	
Smoker						
Never (n=6)	173 (51–397)	NS	1.2 (0.9–2.7)	NS	22.3 (8.5–48.9)	NS
Current/previous (n=6)	274 (85–618)		2.3 (0.6–6.0)		18.7 (14.8–33.0)	
Time from diagnosis (months)						
≤28 (n=7)	245 (51–618)	NS	2.7 (0.8–6.0)	0.09	14.8 (8.5–22.2)	0.007
>28 (n=5)	200 (85–410)		1.1 (0.6–1.6)		33.0 (19.3–48.9)	
Prior therapeutic lavage						
No (n=3)	200 (74–397)	NS	1.0 (0.9–1.7)	NS	14.5 (9.3–37.5)	NS
Yes (n=9)	245 (51–618)		1.6 (0.6–6.0)		19.3 (8.5–48.9)	
Time since lavage (months)						
<6 (n=4)	260 (51–618)	NS	2.7 (0.8–6.0)	NS	18.2 (8.5–22.3)	0.09
≥6 (n=5)	245 (85–410)		1.3 (0.8–3.0)		31.5 (14.8–48.9)	

Values are mean (range).

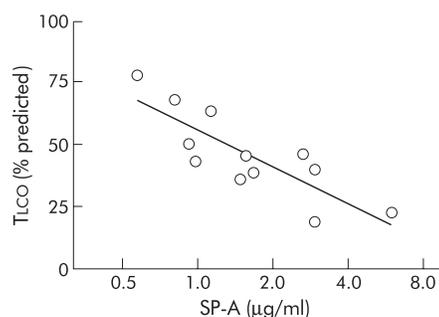
**Table 3** Correlation between pretreatment levels of anti-GM-CSF antibody, surfactant protein (SP)-A, SP-B, serum lactate dehydrogenase (LDH), and other indicators of disease severity

Parameter	Anti-GM-CSF Ab (µg/ml)	p value	SP-A (µg/ml)*	p value	SP-B (µg/ml)†	p value	LDH (%ULN)	p value
Arterial PO <sub>2</sub> (mm Hg) (n=11)	-0.123	NS	-0.704	0.01	-0.247	NS	-0.175	NS
[A - a]DO <sub>2</sub> (mm Hg) (n=11)	0.123	NS	0.715	0.009	0.088	NS	0.025	NS
VC (% predicted) (n=12)	-0.022	NS	-0.646	0.02	0.210	NS	-0.461	NS
TlCO (% predicted) (n=12)	0.017	NS	-0.829	0.0009	0.179	NS	-0.482	NS
LDH (%ULN) (n=12)	0.237	NS	0.462	0.1	0.056	NS		
SP-B (µg/ml) (n=12)†	0.123	NS	-0.321	NS				
SPA (µg/ml) (n=12)*	0.189	NS						

PO<sub>2</sub>=arterial oxygen tension; [A-a]DO<sub>2</sub>=alveolar-arterial oxygen gradient; VC=vital capacity; TlCO=carbon monoxide transfer factor.  
 \*Normal range for serum level of SP-A is <0.25 µg/ml. Values were not normally distributed so analysis was performed following log<sub>10</sub> transformation of data.  
 †Normal range for serum level of SP-B is <2.6 µg/ml. Values were not normally distributed so analysis was performed following log<sub>10</sub> transformation of data.



**Figure 1** Correlation between pretreatment surfactant protein A (SP-A) levels (log transformed) and baseline alveolar - arterial oxygen gradient ([A - a]DO<sub>2</sub>) in 12 patients (R<sup>2</sup>=0.511, p=0.009) with regression line shown.



**Figure 2** Correlation between pretreatment surfactant protein-A (SP-A) levels (log transformed) and baseline carbon monoxide transfer factor (TlCO) in 12 patients (R<sup>2</sup>=0.687, p=0.0009) with regression line shown.

**DISCUSSION**

Recent studies have demonstrated the presence of neutralising antibodies to GM-CSF in the BAL fluid and serum of patients with idiopathic AP.<sup>7-9, 22</sup> These anti-GM-CSF antibodies are likely to be pathogenic. Through the inhibition of endogenous GM-CSF activity, the ability of alveolar macrophages to clear surfactant is grossly impaired,<sup>10</sup> resulting in the characteristic feature of the disease—namely, excess surfactant accumulation in the airspaces. Consistent with such a model, patients with idiopathic AP have an attenuated haematopoietic response to administered GM-CSF, whereas patients with congenital AP, who do not have anti-GM-CSF antibodies,<sup>9</sup> have a normal haematopoietic response to this growth factor.<sup>33</sup>

Although it may have been anticipated that concentrations of the anti-GM-CSF antibody would reflect disease severity and predict response to GM-CSF treatment, we found no such relationship. The reasons for this are unclear but could relate to properties of the quantification method used, which measures the amount of antibody by weight of protein but does not

measure either binding affinity for native GM-CSF or biological potency in neutralising GM-CSF. Prior analysis showed that the anti-GM-CSF antibody is a mixture of IgG, and IgG<sub>2</sub> subclasses<sup>9</sup> which may have different binding affinities or be directed against different epitopes of the GM-CSF molecule. However, serial analysis within individual patients also failed to reflect disease activity accurately, with antibody levels undergoing modest declines in all cases regardless of response. We have previously shown that a therapeutic response to GM-CSF required the delivery of adequate amounts to overcome, at least transiently, the neutralising action of the endogenous antibody as reflected by the development of a modest eosinophilia.<sup>22</sup> This suggests that a bioassay of total GM-CSF neutralising capacity may predict the response to GM-CSF treatment more accurately.

The alveolocapillary membrane of healthy lung facilitates gaseous transfer, while providing a barrier to the movement of fluids and solutes. Although this barrier is normally very successful at partitioning the proteins of the pulmonary epithelial lining fluid, there is some leakage. While a range of proteins

**Table 4** Significance of correlations between serological parameters and conventional measures of disease severity within individual patients during the entire treatment period

	Anti-GM-CSF Ab (µg/ml)		SP-A (µg/ml)		SP-B (µg/ml)		LDH (%ULN)	
	No	p value	No	p value	No	p value	No	p value
[A - a]DO <sub>2</sub> (mm Hg)	98	NS	102	0.054	102	NS	132	0.010
TlCO (% predicted)	69	NS	74	NS	74	NS	100	NS
VC (% predicted)	78	NS	80	NS	80	NS	106	0.019

[A-a]DO<sub>2</sub>=alveolar-arterial oxygen gradient; VC=vital capacity; TlCO=carbon monoxide transfer factor.  
 p values are calculated from the signed rank test for association within patients (see Appendix).

has been shown to leak into the circulation,<sup>35,36</sup> the surfactant proteins have been most extensively studied. Intuitively, circulating levels of the surfactant proteins should be determined both by their rate of entry into the circulation, which is a reflection of the combination of their alveolar levels and the alveolocapillary membrane permeability, and by the rate of systemic clearance. However, systemic clearance is not a major determinant of circulating surfactant protein levels in patients with acute lung injury<sup>37</sup> and is unlikely to be altered in patients with AP. Similarly, there is no evidence of increased alveolocapillary membrane permeability in these patients, so the increase in circulating SP-A and SP-B levels is likely primarily to reflect their increased alveolar levels.<sup>17</sup>

Surprisingly, in the present study we found SP-A to be a better marker of disease severity than SP-B. This is contrary to our previous experience in other forms of lung disease where SP-B was a more sensitive marker, probably due to its smaller size (~18 kDa v ~650 kDa).<sup>24</sup> We do not know the exact structural nature of the surfactant proteins that leaked into the circulation, nor their route of entry, and can only speculate as to why SP-A appears to be a more sensitive marker of disease in patients with AP.

Kuroki *et al*<sup>23</sup> reported that the levels of SP-A were raised in all 11 patients with AP studied and declined after lavage in the one patient evaluated. Serum levels of SP-D were also raised in all 14 patients with AP studied.<sup>25</sup> A decline in serum levels following lavage has been reported in four instances,<sup>20</sup> but in a group of patients with various interstitial lung diseases (including AP) no correlation was found between SP-D levels and other pulmonary function parameters.<sup>20</sup> This clinical trial is the first to evaluate serum SP-B levels in patients with AP.<sup>22</sup>

Although it is a simple and readily available test, LDH levels in patients with AP have only been reported in small series to date. The isoenzyme pattern of LDH in serum and BAL fluid shows an increase in all isoenzymes,<sup>27</sup> which indicates that the cellular source is likely to be heterogeneous. It is probable that the cellular turnover of alveolar macrophages is one source of the raised LDH level, although a predominant LDH<sub>1</sub> increase would be expected if this were the dominant source.<sup>38</sup> Types I and II pneumocytes as well as bronchial epithelial cells are other possible sources.<sup>27</sup> There were 75 published English language reports up to 1997 of patients with AP with data on LDH levels, and these were raised in 85%.<sup>39</sup> Simultaneous values for LDH and [A - a]Do<sub>2</sub> were reported in 24 of these cases and were highly correlated ( $R=0.699$ ;  $p=0.0001$ ),<sup>39</sup> consistent with the results of our analysis. Similarly, LDH levels have been reported to decline following lavage.<sup>27</sup> We also found that LDH was correlated with serum SP-B levels, suggesting that it may be an indirect indicator of total lung surfactant accumulation.

Protein products of pulmonary epithelial cells including carcinoembryonic antigen (CEA)<sup>40</sup> and the mucin KL-6<sup>41,42</sup> have also been found to be increased in the serum of patients with pulmonary AP and suggested as potential markers of disease severity. The levels of CEA were significantly correlated with [A - a]Do<sub>2</sub> and serum LDH, and declined following successful therapeutic lavage in the three cases examined serially.<sup>40</sup> In the current series CEA was measured in three patients and was moderately increased in two. KL-6 has also been reported to decline following lavage in the two cases reported.<sup>41,42</sup>

In conclusion, our study indicates that serum levels of SP-A reflect disease severity in patients with AP before GM-CSF treatment. Serum levels of LDH most accurately reflect the dynamic changes in disease severity during treatment. Despite the importance of the presence of anti-GM-CSF antibodies in identifying patients with AP, serum levels did not reflect disease severity.

## APPENDIX: MODIFIED SIGN TEST FOR TESTING FOR ASSOCIATION WITHIN PATIENTS

Suppose there are  $n$  patients and  $n_i$  pairs of observations ( $x_i, y_i$ ) recorded for the  $i$ th patient ( $i = 1, 2, \dots, n$ ). It is desired to test whether

there is an association between these two variables within patients. To avoid problems related to repeated measurements, a summary measure of association is calculated for each patient, here the slope of a straight line fitted to the variables ( $y$  versus  $x$ ). In applications in this paper the  $y$  variable is taken to be the serological measure and the  $x$  variable the conventional measure of disease severity. However, the same test result ensues if these roles are reversed. Let  $b_i$  be the slope of the fitted straight line of  $y$  versus  $x$  for data on the  $i$ th patient. The test statistic is:

$$T = \sum_{i=1}^n Z_i w_i |b_i|$$

where  $Z_i$  ( $i=1, 2, \dots, n$ ) are independent binary random variables taking values  $-1$  or  $+1$  with equal probabilities,  $w_i$  are weights (see below), and  $|b_i|$  are the absolute values of the estimated slopes. Under the null hypothesis of no association between the variables, given the absolute values of the slopes, the sign of each slope could, with equal likelihood, be positive or negative. The observed value of the test statistic,  $t$ , is:

$$t = \sum_{i=1}^n w_i b_i$$

which is compared with the extremes of the exact null hypothesis distribution of  $T$ . The  $p$  value is:

$$p = p[|T| \geq |t|]$$

Because the numbers of observations vary between patients, as do the ranges of the variables involved, it is appropriate to give more weight to estimated slopes from patients with more observations and wider ranges. The weight chosen for the analyses in this paper is:

$$w_i = \sum_{j=1}^{n_i} (x_{ij} - \bar{x}_i)^2$$

because, in conventional straight line regression, the variance of the slope estimator is inversely proportional to this quantity.

A modification of this procedure is to use the ranks of the quantities,  $w_i |b_i|$ , i.e.

$$T_R = \sum_{i=1}^n Z_i \text{rank}(w_i |b_i|)$$

to produce a signed rank test and that is in fact the test used in this paper. Results, however, were similar whether ranks or raw scores were used.

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