Eur Respir J 2003; 21: 414–420
DOI: 10.1183/09031936.03.00060902
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European Respiratory Journal
ISSN 0903-1936

Changes in bronchoalveolar lavage fluid proteins in sarcoidosis: a proteomics approach

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Changes in bronchoalveolar lavage fluid proteins in sarcoidosis: a proteomics approach. F. Sabounchi-Schütt, J.Åström, U. Hellman, A. Eklund, J. Grunewald. ©ERS Journals Ltd 2003.

ABSTRACT: In sarcoidosis, an inflammatory lung disease, the protein profile of bronchoalveolar lavage fluid (BALF) is altered. To study the BALF protein pattern changes in sarcoidosis, samples from six patients and four healthy individuals were analysed by two-dimensional polyacrylamide gel electrophoresis.

A comparison of the protein-spot patterns showed a significantly higher number of protein spots in the pH range 5.5–6.7 in patients compared to controls (472 *versus* 384). Furthermore, the number of protein spots in the patients were significantly decreased in the acidic pH range 4.5–5.5 (399 *versus* 518). Measurement of the optical density in the gels showed varying expression levels for several protein spots. Seventeen of the altered protein spots were identified, of which seven have previously not been reported for BALF. Many of these are nonplasma proteins involved in the inflammatory and oxidant-antioxidant processes.

In conclusion, the bronchoalveolar lavage fluid protein content is altered in sarcoidosis patients, especially for proteins that are not derived from plasma. The described proteomics approach will in the future be used to asses overall changes in the protein content associated with sarcoidosis and may offer the possibility of identifying disease-specific proteins.

Eur Respir J 2003; 21: 414–420.

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Keywords: Bronchoalveolar lavage fluid, matrix-assisted laser desorption/ionisation time of flight mass spectrometry, protein quantitation, protein-spot pattern, sarcoidosis, two-dimensional polyacrylamide gel electrophoresis

Received: July 9 2002
Accepted after revision: November 29 2002

This study was supported by the Swedish Medical Research Council (K2002-74X-14182-01A), The Swedish Heart-Lung Foundation, the King Oscar II Jubilee Foundation and Karolinska Institutet.

Sarcoidosis is a systemic granulomatous disease that primarily affects the lungs and the lymphatic system. The cause of the disorder is still unknown. However, since sarcoidosis was first described, much progress has been made in terms of understanding the clinical and unique immunological and pathological features of the disorder. Less is known about the protein specificity and different variants of the proteins, which may contribute to the development and expression of the disease. A classic tool for studying epithelial lining fluid (ELF) proteins is bronchoscopy with lung lavage. Sampling ELF by bronchoalveolar lavage (BAL) has permitted the recovery of airway alveolar space cells and soluble substances in the extracellular lining fluid, which have been used both diagnostically and as research materials. The retrieval of soluble substances and secretory proteins *via* BAL has contributed to the understanding of the pathogenesis of sarcoidosis and other inflammatory lung disorders [1–3]. Numerous studies have described the cellular components of the BAL fluid (BALF) in sarcoidosis [1, 4, 5]. However, the overall distribution of the proteins in human BALF is still not well known.

Recent studies on BALF suggest that two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) may be useful for investigation of changes in protein expression of patients with different lung diseases [6–9]. It is a powerful method capable of displaying the protein distribution in an organ without the requirement of detailed knowledge of individual proteins. This method enables protein separation from complex biological samples, such as BALF, resulting in a single gel with a hundred to thousands of protein spots resolved in it. The current authors have previously reported the detection and identification of BALF-specific proteins from healthy individuals [10]. The goal of the current study was to establish an overall pattern of soluble BALF proteins in sarcoidosis and to investigate any changes in protein expression associated with this disease. To do this 2-D PAGE in combination with narrow-range pH gradients has been used. The narrow-range pH gradients maximises the resolving power, thereby reducing the number of co-migrating protein spots and facilitates the subsequent identification procedure by peptide-mass fingerprinting using mass spectrometry (MS). In order to improve the detection of low-abundance proteins and to enable protein identification, the recently developed paper bridge loading method [11] was used. Six sarcoidosis patients with common clinical features, *i.e.* Löfgren′s syndrome [12], and four healthy controls were included in this study. Besides a detailed characterisation of the BALF proteins in these individuals, total and differential cell counts were also performed.

Materials and methods

Study population

BALF from six patients with sarcoidosis and four healthy individuals was investigated. All individuals were recruited

from the Dept of Medicine, Karolinska Hospital (Stockholm, Sweden) with the approval of the ethical committee and informed consent from the patients and the healthy individuals. All patients had an acute onset of sarcoidosis with Löfgren′s syndrome [12], including bilateral hilar lymphadenopathy, fever, ankle arthralgia and/or erythema nodosum, *i.e.* a clinical picture strongly in support of the diagnosis. Furthermore, all patients had an elevated CD4/CD8 BALF ratio (table 1) in accordance with the diagnosis and three of them had in addition a positive biopsy (patient nos. 3, 4 and 5). The healthy individuals had normal chest radiography and were without any signs of respiratory diseases and other inflammatory or infectious disorders.

Sample preparation

Bronchoscopy with BALF was performed as previously described [13]. Briefly, under local anaesthesia, the flexible fibreoptic bronchus (Olympus Optical Co. Ltd, Tokoyo, Japan) was wedged in the right middle lobe bronchoscope and five aliquots of 50 mL sterile 37°C buffered saline was instilled. The fluid was gently aspirated after each aliquot, collected in a sterile siliconised bottle and kept on ice. The lavage was then filtered through a single layer of Dacron net type AP32 (Millipore, Sundbyberg, Sweden) and cells were separated by centrifugation at 400×*g* for 10 min at 4°C. The cell pellet underwent cytospin centrifugation at 500×*g* for 3 min (Cytospin 2; Shandon, Runcorn, UK) followed by manual differential counting of cells after staining with May-Grünwald and Giemsa solution. The BALF supernatant was kept on ice and stored at -80°C until used. The BALF was thawed and the protein concentration was determined using a protein assay kit, based on the method described by BRADFORD [14] with bovine serum albumin as the reference. In order to concentrate the proteins, remove salt and low molecular-weight components, the fluid was applied to an Oasis HLB extraction cartridge (Waters Co., Milford, MA, USA), equilibrated with 0.1% trifluoroacetic acid (TFA). After washing the cartridge with 10% acetonitrile (ACN), 0.1% TFA, the proteins were eluted in 80% ACN, 0.1% TFA. The eluted protein was evaporated to dryness using a vacuum centrifuge (Savant Speed-Vac concentrator; Thermo Electron Corporation, Waltham, MA, USA). In order to equalise the amount of the total protein used, each BALF sample after preparation and lyophilisation was weighed and exactly the same amount in g was loaded for the first dimension separation of each sample. For each experiment (analytical) the same amount (20–40 μg) was used. The variation of 20–40 μg was dependent on the pH interval used, but an equal amount was used for every comparison in the different pH intervals. A higher amount of protein (2 mg) was loaded for the mass spectrometry identification experiments. The protein pellet was resuspended in a solution containing 8 M urea, 4% 3[(3-cholaminopropyl) diethylamonio]-1-propane sulphonate (CHAPS), 65 mM dithiothreitol (DTT), and 2% of the 3–10 nonlinear immobilised pH gradient (IPG) buffer, to the desired concentration.

Two-dimensional electrophoresis

Immobilised dry strips (Amersham Biosciences, Uppsala, Sweden) pH 3–7, 4–7, 6–9, 5.5–6.7 and 4.5–5.5 18-cm long were rehydrated overnight at room temperature in a solution containing 8 M urea, 2% CHAPS, 1% of the related IPG-buffer, 18 mM DTT and a trace of bromophenol blue. The sample was applied in a volume of 60 μL (20–40 μg) using cup at the anodic end of the strips. The proteins were focused on electrophoresis units, at 19°C, using a one-step linear voltage gradient increasing from 300–3,500 V in 2 h, the voltage was then kept constant at 3,500 V for an additional 18 h, giving a total of 65 kVh. The strips were equilibrated for 15 min in 19 mM DTT, 50 mM tris, 6 M urea, 30% glycerol, 2% sodium dodecylsulphate, and then for 15 min in the same solution except that DTT was replaced by 0.2 M iodoacetamide. The second-dimension separations were performed as described previously [10]. The gels were silver stained with automated silver staining equipment. The method for staining was as previously reported [15]. Briefly, after fixation with acid and sensitisation with sodium thiosulphate in alcoholic sodium acetate, the gels were impregnated with silver nitrate and developed by reduction of silver ion using sodium carbonate solution containing formaldehyde. For the micropreparative experiments 2 mg BALF proteins were separated using the cup loading or the recently developed paper bridge application method [11]. Briefly, 0.5 mL sample solution was added to a thick filter paper. The filter paper was positioned as a bridge between the acidic end of the IPG strip and the corresponding electrode. The proteins were focused on the electrophoresis unit for a total of 100 kVh. After second-dimension separation the gels were stained with Coomassie brilliant blue R 250. The stained second-dimension gels were scanned in an ImageScanner™ (Amersham Biosciences). Parallel experiments were performed for each pH gradient. The analytical gel figures are composites of two parallel experiments from each subject; 13 cm in the focusing direction from one gel and 5 cm from the other. The region of overlap has been removed and the point of assembly is visible.

Table 1. – Summary of the individual characteristics of the 10 individuals included in this study

	Patients						Healthy controls			
	1	2	3	4	5	6	7	8	9	10
Age yrs	31	32	25	37	40	39	28	24	49	25
Sex	M	M	F	M	M	M	F	M	M	M
Smoking	Ex	Ex	No	No	No	No	No	No	No	No
Radiographical stage	I	I	I	I	II	II	0	0	0	0
Cell concentration $\times 10^6$ L^{-1}	192	146	164	296	393	400	89	60	134	122
CD4/CD8 ratio	14	4.2	8.0	9.8	11	8.0	3.3	0.9	ND	1.6
BAL cell differential counts %										
Alveolar macrophages	98	87	86	51	51	53	96	84	98	92
Lymphocytes	2.2	13	13	46	49	46	2.8	15	1.6	6.2
Neutrophils	0.2	0.4	1.4	2.0	0.6	0.2	0.4	0.8	0.4	1.2
BALF protein concentration $\mu g \cdot mL^{-1}$	136	168	124	98	198	131	80	72	64	51

BAL: bronchoalveolar lavage; BALF: bronchoalveolar lavage fluid; M: male; F: female; Ex: exsmoker; No: nonsmoker; ND: no data.

Evaluation of the gel images

The resulting gels were analysed using ImageMaster 2-D software version 3.01 (Amersham Biosciences) with a computerised 8-bit system and freshly calibrated. The typical analysis parameters for automatic spot detection were as follows: sensitivity was set to 9,636, operator size to 73, noise factor to 5, background factor to 68 and split level to 7. The gels were manually edited. Since all gel images contain levels of background intensity inherent in the scanned image, the background was subtracted by the mode of nonspots for the accurate spot measurements. To measure the optical density (OD) of the protein spots detected in the silver stained gels the volume of each spot was divided by the total volume of all of the spots of the same gel. Since this method of normalisation tends to produce extremely small values, the result was multiplied by a scaling factor of 100, which produces spot percentage volume. This volume per cent is dependent on the saturation of the spots with respect to the staining differences. The software was set to not generate autoseeds (*i.e.* spots common on both gels), but to allow for positioning of user seeds. In order to detect differentially expressed protein spots in the different gels, by instruction, the software created one map containing all spots detected in the four gels of the healthy controls and presented this as a "reference map". The software used this reference map only as a start point for the comparison between patients and the controls. For detailed comparisons between individual patients and controls, the individual gels of the patients were subsequently compared to each individual control gel (four gels). Only those spots that were reproducibly different in the patients compared to all the controls were considered. The total number of spots, nonmatched spots and the normalised volume percentage expressed in OD value were then calculated for each gel. The spots that differed with $\geqslant 50\%$ in intensity and those not matched to the control gels were then highlighted and inspected carefully for inappropriate matching, staining artifacts or bad spot detection.

Protein identification

The selected gel plugs were punched out from the Coomassie stained gels. Removal of salts and staining residues was performed as described previously [10]. The gel pieces were then dried under vacuum and rehydrated in a solution containing 5 μL trypsin (0.3 μg) overnight at 30°C. The generated peptides were extracted, using 100 μL of 50% ACN, 0.45% TFA and incubated twice at 30°C for 1 h. The pooled extracts were then lyophilised for 2 h. The lyophilised sample was dissolved in 5 μL of matrix (a saturated solution of α-cyano-4-hydroxy-cinnamic acid, (Fluka, Cork, Ireland) in 50% ACN, 0.45% TFA containing reference peptides, ^{ile7}angiotensin III, human adrenocorticotropin 18–39 (Sigma, Cork, Ireland). Finally, 1 μL was applied onto the target slide using the dried droplet method [16]. For low concentration peptides ZipTip clean-up (Millipore) was used according to the manufacturer's instruction. Mass spectra were acquired using matrix-assisted laser desorption/ionisation time of flight (MALDI-ToF) mass spectrometers, Ettan MALDI-ToF (Amersham Biosciences) or Bruker Autoflex (Bruker Daltonics, Bremen, Germany) as described previously [17]. The spectra were calibrated using the internal reference peptides or trypsin autodigestion peaks. Trypsin autodigestion peaks and internal reference peptides were excluded from the database searching. The search program proFound version 4.10.4 was used for peptide mass fingerprinting [18]. The parameters for the searches were as follows: species of origin, all taxa or Homo sapiens; molecular range set to 5–3,000 kDa; isoelectric point (pI) range from 1–14; and digestion by trypsin allowing for no more than one missed cut. Monoisotopic peptide masses were used and the mass tolerance error was set up to 0.1–0.2 Da. Cysteine was considered as carboxyamidomethyl cysteine and partial modification of methionine by oxidation was allowed. The first listed sequence entry of the top protein candidate is shown in table 2. Spot identifications giving a Z score (a statistical value of the quality and the reliability of the search

Table 2. – Summary of the bronchoalveolar lavage fluid proteins from pH ranges 4.5–5.5 and 5.5–6.7 identified using matrix-assisted laser desorption/ionisation time of flight mass spectrometry and peptide mass fingerprinting

Spot no. #	Protein name	Database *i.d.*¶	Score Z value+	Coverage %§	OD intensity in patients
1	Not determined				Increased*
2	Monocyte/neutrophil elastase inhibitor	Gi 266344	1.50	19	Decreased*
3	Unknown				Decreased**
4	Calcyphosine	Gi 4757908	2.30	52	Decreased*
5	Glutathione *S*-transferase, chain A	Gi 11514451	2.19	62	Decreased**
6	Thioredoxin	Gi 230939	0.91	35	Decreased
7	Immunoglobulin κ light chain	Gi 2894829	1.48	40	Increased*
8	Human β_2 glycoprotein I	Gi 6435718	2.29	18	Increased
9	Unknown				Increased**
10	Clara cell	Gi 256397	0.80	19	Increased
11	β-galactosidase binding lectin	Gi 4504981	1.14	45	Decreased**
12	Aldehyde dehydrogenase 1	Gi 4502031	0.78	14	Decreased*
13	Cathepsin H	Gi 29708	0.77	17	Decreased*
14	Annexin I	Gi 4502101	2.31	29	Decreased**
15	Antioxidant protein II	Gi 4758638	2.41	48	Decreased**
16	Fatty acid binding protein	Gi 119781	2.40	69	Decreased**
17	Calgizzarin	Gi 5032057	1.10	41	Decreased*
18	Not determined				Decreased*
19	Heat shock 27 kd protein 1	Gi 4504517	2.38	47	Increased
20	Aldehyde reductase	Gi 1633300	2.30	31	Decreased
21	β_2-microglobulin	Gi 195503	2.14	60	Increased

OD: optical density. #: spot no. refers to the annotation in figures 1a and b; ¶: National Center for Biotechnology Information (NCBI); +: score Z value shows the quality of database search results; §: sequence coverage refers to the observed sequence coverage of the assigned protein. *: $p<0.05$, from the comparison of the six patients and four healthy controls; **: $p<0.01$, from the comparison of the six patients and four healthy controls.

result [18]) <0.5 in the table are marked as "not determined", since their identifications were regarded to be uncertain.

Statistical methods

To be regarded as differentially expressed, only protein spots with OD values that differed by >50% in at least five of the six patients compared to the reference map were considered. All OD values of differentially expressed protein spots of individual patients were thereafter compared to all the corresponding control values. Differences between patients and controls were tested using the nonparametric Mann-Whitney U-test and a $p<0.05$ was considered significant. Results are presented as median with p25 and p75 values (tables 1 and 3) as the range.

Results

Characteristics of the investigated individuals

Individual characteristics and the results of the differential cell counts of the 10 BALF samples included in this study are shown in table 1. Patients BALF compared to control samples generally contained a higher concentration of cells, more relative numbers of lymphocytes and significantly elevated CD4/CD8 ratios. The total protein concentration in BALF was significantly elevated ($p<0.01$) in the patients (median 134 $\mu g \cdot mL^{-1}$ (range 124–168)) compared to controls (68.0 $\mu g \cdot mL^{-1}$ (57.5–76.0)).

Bronchoalveolar lavage fluid protein-spots patterns differ in sarcoidosis patients compared to healthy individuals

To investigate the protein expression pattern in the disease states of human BALF, 2-D (PAGE) separations were run. Initially, wide ranging pH gradients 3–7, 6–9 and 4–7 were used for separation of BALF proteins in the first dimension. For each pH interval at least two parallel experiments were performed. From the 2-D protein patterns obtained, a high number of protein spots in the pH region 4.5–6.7 were distinguished, approximately one-fifth of the spots were found in the pH region 6–9, while only a limited number of spots were detected below pH 4.0 (data not shown). This was in agreement with a previous report on the spot distribution in the normal BALF map [10]. Based on these observations the authors decided to focus the proteomics BALF investigation on the pH interval 4.5–6.7, using narrow range IPG strips 4.5–5.5 and 5.5–6.7. Representative examples of BALF protein gels in these pH regions from sarcoidosis patients are shown in figure 1.

Table 3. – The number of protein spots detected by two-dimensional polyacrylamide gel electrophoresis separation of bronchoalveolar lavage fluid from the patients and the healthy control in the pH-interval 4.5–5.5, 5.5–6.7, and in the combined pH interval 4.5–6.7

	Patients						Healthy controls			
	1	2	3	4	5	6	7	8	9	10
pH 4.5–5.5	464	407	392	329	418	359	517	454	550	577
pH 5.5–6.7	491	440	778	411	453	615	367	296	430	402
pH 4.5–6.7	955	847	1170	740	871	974	884	750	980	979

In figure 1 a region of heavy staining can be seen in the pH region 5.5–5.8, which corresponds to the migration of albumin in the gels. Several of the detected protein spots have previously been identified and characterised (fig. 1, [10]).

In the control BALF, more protein spots appeared in the acidic pH range 4.5–5.5 compared to the pH interval 5.5–6.7, while in the patient BALF such a trend was not obvious (table 3). There were significantly more spots in the pH range 5.5–6.7 in the patient compared to controls (472 (440–625) *versus* 384 (331–416); $p<0.05$), while in the acidic pH interval 4.5–5.5 the number of spots were significantly decreased in the patients samples (399 (359–418) *versus* 518 (485–598); $p<0.05$) (table 3).

Variation of the protein expression level in patients versus *controls*

To quantify the protein expression level, differences between patient and control 2-D gels were carefully inspected. Spots that reproducibly differed in intensity, and for which a reliable OD could be calculated, were selected for further

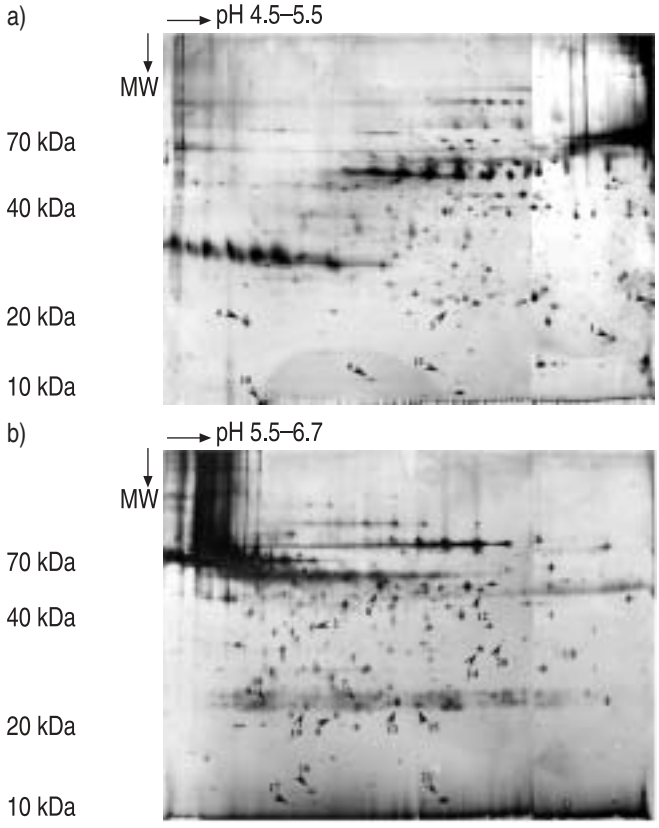

Fig. 1. – The two-dimensional protein-spot pattern of sarcoidosis bronchoalveolar lavage fluid (BALF) in the pH interval a) 4.5–5.5 (patient 2) and b) 5.5–6.7 (patient 5). A 30 µg BALF sample was separated in immobilised pH gradient 4.5–5.5 or 5.5–6.7 strips. The second dimension separation was run using 9–18% gradient sodium dodecylsulphate-polyacrylamide gel electrophoresis. The resulting protein patterns were visualised using silver staining. Arrows indicate identified proteins altered in the patients. The numbers refer to the identification of the proteins presented in table 3. Since a second dimension gel is only 13-cm wide and the strips of the first dimension are 18 cm, the gel images are composites of two parallel experiments from the same patient, where 13 cm in the focusing direction is from one gel and 5 cm from the other. The region of overlap has been removed and the point of assembly is visible.

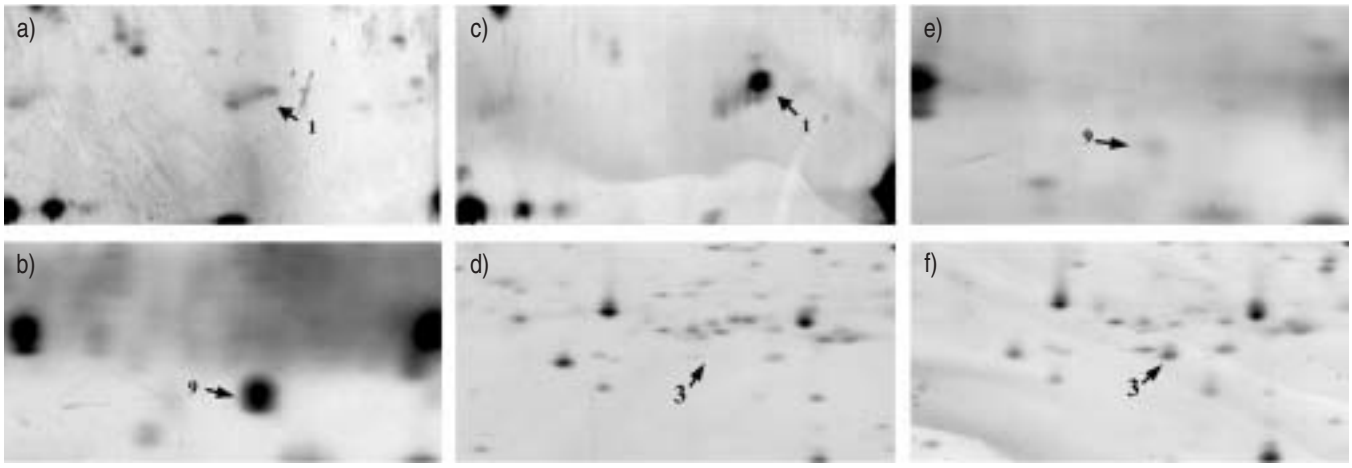

Fig. 2. – Enlarged regions of the two-dimensional gel images of bronchoalveolar lavage fluid (BALF) samples showing protein spots 1 (a, c), 9 (b, e) and 3 (d, f). a), d) and e) show the pattern in healthy controls while b), c) and f) show the same region in the sarcoidosis BALF. The spots are indicated by arrows and the numbers refer to the identification of the proteins presented in figure 1 and table 3.

analysis. For each of these spots at least two independent OD measurements were obtained in the same pH interval, and in most cases additional OD values were acquired from overlapping (wide or narrow range) pH intervals. In total, 21 spots were found to differ between patients and controls (annotated in fig. 1). In figure 2 typical spot pattern variations are highlighted, showing qualitative differences between controls and patients.

Fifteen of the altered protein spots differed significantly. The OD values for these proteins are shown in figures 3a and b. Of the 21 altered protein spots in the patients, fourteen were decreased and seven were increased in intensities compared to the healthy controls. In the pH range 5.5–6.7 fourteen protein spots were changed, among them nine were decreased while five were increased in intensity, compared to the controls. Seven of the altered protein spots were in the pH range 4.5–5.5, three of five were decreased and two were increased. Sixteen of the altered protein spots had molecular weights between 10–25 kDa and 12 of these were decreased in intensity.

Identification of proteins in bronchoalveolar lavage fluid

In order to identify the altered proteins in the patients, all the 21 protein-spots were subjected to MALDI ToF MS. Seventeen of these proteins could be identified (table 2). Figures 1a and b show the location of these proteins in the 2-D maps of the sarcoidosis patients. Seven of the identified proteins have, to the best of the authors′ knowledge, not previously been recognised in published BALF 2-D maps: monocyte neutrophil elastase inhibitor (spot 2), calcyphosine (spot 4), β-galactosidase binding lectin (spot 11), aldehyde dehydrogenase I (spot 12), cathepsin H (spot 13), antioxidant protein II (spot 15) and aldehyde reductase (spot 20). The positions of spots 5, 11 and 12 have, in addition, been verified against the spot positions found in other 2-D maps [19, 20]. Two proteins (spot 3 and 9) were assigned as unknown by the database. Spots 1 and 18 were marked as "not determined" in table 2, since their spot identifications gave a Z score <0.5, indicating uncertain identifications.

Discussion

The patients included in this study were chosen according to their diagnosis of acute sarcoidosis with Löfgren′s syndrome. This group is characterised by similarities in their clinical features and in their immunological response [12, 21, 22]. The BALF cellular composition of these patients showed,

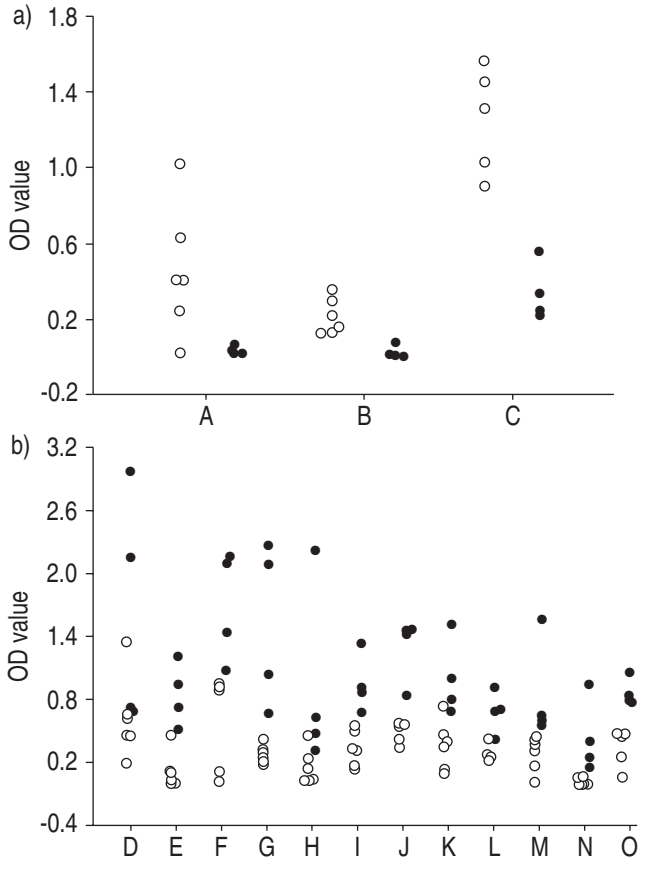

Fig. 3. – Optimal density (OD) values of proteins a) upregulated and b) downregulated in the patients. A: unknown identity; B: not determined; B and C: immunoglobulin κ light chain; D: (calcyphosine); E: β-galactosidase binding lectin; F: cathepsin H; G: fatty acid binding protein; H: calgizarrin; I: glutathione *S*-transferase; J: antioxidant proteins II; K: aldehyde dehydrogenase I; L: neutrophil elastase inhibitor; M: annexin I; N: not determined; O: unknown identity. D–H were grouped as proteins involved in cell-cell interactions and proliferation processes. I–K as proteins involved in oxidation, antioxidatory processes. L, M as proteins involved in anti-inflammatory processes. N and O are unknown. ○: patients; ●: controls. All differences were statistically significant ($p<0.05$).

as expected, an increased CD4/CD8 ratio [23] and an increase of the relative number of lymphocytes. Although the total number of protein spots in BALF of the patients was approximately equal to that of the healthy controls, the total protein concentration was significantly increased. This could be due to an increase of some plasma proteins in BALF of sarcoidosis in agreement with some published reports [9, 24]. An increase of a few individual proteins would influence the total protein concentration in the samples but not necessarily affect the number of the protein spots detected in a 2-D gel. An overview of BALF protein alterations in different lung diseases has been difficult to obtain since the BAL technique produces considerable dilution of proteins and a very high salt content in the retrieved fluid. The composition of BALF proteins may also vary in different levels of the respiratory tract. Furthermore, many proteins in BALF are either present at a low concentration or co-immigrate during the electrophoresis focusing. Here, an overview of the total protein distribution of six sarcoidosis patients with distinct clinical similarities in the pH interval 4.5–6.7 is presented. Narrow-range pH gradients were used, which allowed a higher sample load, thereby enabling the detection of more protein spots and isoforms than with previously used wide range pH gradients. The paper bridge sample application method was also used [11], which allowed for the use of high sample load, the detection of low-abundance proteins, the separation of high molecular-weight proteins and improved spot resolution. This approach allowed for the detection and identification of seven proteins, which previously have not been reported in the available 2-D maps of BALF.

The overall protein composition in the sarcoidosis map shows a high rate of similarity to the corresponding control map. As in the control maps, most of the protein spots appear in pH interval 4.5–6.7. However, in patients, the number of proteins in the pH interval 5.5–6.7 were significantly elevated compared to the same region in the control samples, suggesting that many of the disease-related proteins could be found in this pH interval. In accordance with this, fourteen of 21 altered protein spots were in this pH interval. In the more acidic region of the sarcoidosis gel, the number of spots decreased significantly compared to the control gel. Interestingly, all of the altered proteins identified in this region were nonplasma proteins, restricted to BALF.

To be able to calculate a reliable OD value for quantitative comparisons, identical samples from the same individual were run several times with different IPGs in the first dimension separation. In addition, parallel experiments with samples from both patients and controls were performed. Moreover, the variability in the silver staining of 2-D gels has been considered through a normalisation of the spot patterns in each gel, before the matching procedure. However, due to 2-D PAGE limitations, the characterisation of all protein spots, which apparently differed in intensity in the present experiments, was not possible, *e.g.* extreme high or low-abounded proteins. Such protein spots either gave an uncertain OD value or had a very low concentration and were absent in the Coomassie gels and therefore could not be subjected to MS.

As many as 12 proteins were significantly decreased in intensity in the patients compared to healthy controls. These proteins included antioxidant proteins. Antioxidant proteins can be expected to provide an initial defence against inhaled toxins, and are also a part of the host defence against a wide range of organisms and may be consumed during the acute sarcoidosis inflammation. The decreased level of these proteins in the patients could be an indicator of imbalance of oxidant/antioxidants in this disease. Furthermore, two proteins identified in this study also involved in oxidative activities (aldehyde dehydrogenase I and aldehyde reductase, which are functionally and structurally related enzymes) were found to be decreased in the sarcoidosis patients.

Lower respiratory tract secretion contains neutrophil elastase [25]. One of the newly identified proteins presented in this work is neutrophil elastase inhibitor (NEI), which regulates the activity of neutrophil elastase, as well as protease, cathepsin G and proteinase. The likely role of NEI is to regulate protease activity and prevent tissue damage by phagocytic cells [26, 27]. Thus NEI-mediated defence against free NE in the lower respiratory tract is significantly decreased in sarcoidosis. The reduced level of annexin I could also reflect the NEI-mediated defence against free NE in the patients. Annexin I, normally present at high concentration on the epithelial surface of the normal lung and in BALF, has potent anti-inflammatory activity [28]. Degradation of this protein has been observed in BALF from patients with various lung diseases [29, 30]. This degradation has also been suggested to be mediated by neutrophil elastase in ELF [31, 32], suggesting a possible link between annexin I and NEI.

Among the decreased proteins in BALF of the patients, a calcyphosine was found, which is involved in cell growth and differentiation and may regulate essential cell functions like proliferation and differentiation as well as cell degranulation [33]. Decreased levels of this protein might cause inhibition of these processes.

A few studies have been performed on human total BALF proteins in sarcoidosis using proteomics [9, 34, 35]. Only one of these studies directly compared BALF of sarcoidosis patients to that of healthy controls [9]. Previous studies have instead focused on establishing and updating the human BALF protein map. WATTIEZ *et al.* [9] reported several proteins to differ in expression in sarcoidosis compared to healthy controls. Although a few of these proteins were also recognised in the present study (*i.e.* β_2-microglobulin and clara cell protein), the majority of the differently expressed proteins identified were not the same. Different experimental conditions, such as using the narrow-range pH gradients for the first dimension separation in this study, precluded any exact comparison to their results and may have been misleading. In this study, an overview of BALF proteins in sarcoidosis is shown and the alteration of 21 silver-stained protein spots is reported; 17 could be identified. Twelve of the 17 identified proteins were significantly reduced in the patients. An increase in the plasma protein level in the patients in the pH interval used was not observed. The majority of the identified proteins were rather acidic ($4.5<pI<6.7$) had low molecular weights (<30 kD) and did not originate from plasma. Furthermore, seven new nonplasma proteins, not reported previously for BALF 2-D maps, are presented. Proteins from two spots did not match with any known protein and were assigned as unknown. Consistent with the effect of the inflammatory processes some of the altered proteins observed in this report are involved in oxidative, anti-inflammatory processes and in the regulation of a variety of cellular processes related to proliferation. However, the exact extracellular role of some identified proteins in this work are presently unknown.

The proteomics approach used in this study will contribute to the characterisation of protein patterns, allow for the assessment of overall changes in the protein content associated with different lung diseases and also for the detection and identification of proteins in bronchoalveolar lavage fluid that might be used as disease-specific markers.

Acknowledgements. The authors would like to thank B. Bjellqvist for interpretation of the 2-D analyses. They would also like to thank S. Gripenbäck for help with 2-D experiments and A-C. Bergman for discussion concerning mass spectrometry analyses.

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