

Comparative Proteomics of Nasal Fluid in Seasonal Allergic Rhinitis

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A comparative proteomic approach was applied to examine nasal lavage fluid (NLF) from patients with seasonal allergic rhinitis (SAR, $n = 6$) and healthy subjects (controls, $n = 5$). NLF samples were taken both before allergy (pollen) season and during season, and proteins were analyzed by two-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) after tryptic cleavage. Twenty proteins were selected and quantified. During allergy season, the levels of six sialylated isoforms of PLUNC (palate lung nasal epithelial clone) were lower in SAR patients than controls, as were the levels of six isoforms of von Ebner's gland protein (VEGP), including a previously undescribed form with N-linked glycosylation, and of cystatin S. PLUNC is a new innate immunity protein and VEGP and cystatin S are two endogenous proteinase inhibitors. By contrast, the levels of an acidic form of alpha-1-antitrypsin were higher in SAR patients than controls. One previously unidentified NLF protein was found in all samples from the SAR patients during allergy season but not in any sample before allergy season: this protein was identified as eosinophil lysophospholipase (Charcot-Leyden crystal protein/galactin 10). MS/MS analysis of the N-terminus of the protein showed removal of Met and acetylation of Ser. Altogether, these findings illustrate the potential use of proteomics for identifying protein changes associated with allergic rhinitis and for revealing post-translational modifications of such new potential markers of allergic inflammation.

Keywords: nasal fluid • proteomics • allergic rhinitis

Introduction

Allergic rhinitis and allergic asthma are very common diseases in western lifestyle countries and a further increase in prevalence is commonly observed.¹ For example, in 1994, there were an estimated 39 million human subjects in the US suffering with allergic rhinitis, accounting for an estimated 1.2 billion dollars in total costs,² and these costs are now even higher.³ Moreover, several studies have demonstrated that allergic rhinitis is a risk factor for allergic asthma^{4,5} and that rhinitis may appear before or in the very early stages of asthma.¹ There are reasons, therefore, to clarify the molecular mechanisms underlying allergic rhinitis and to develop new means by which this disorder can be early diagnosed and efficiently treated. In seasonal allergic rhinitis (SAR), there is a hyper-response to outdoor environmental allergens such as pollen: these allergens bind to preformed IgE on nasal mucosal mast cells and basophils and cause the release of proinflammatory

mediators with ensuing recruitment and activation of a variety of inflammatory cells. Thus, SAR is associated with nasal mucosal inflammation involving mast cells, basophils, eosinophils, lymphocytes, and their mediators, which are well documented.^{6–7} Yet, it cannot be ruled out that other, hitherto unidentified mediators may play important parts.

To identify inflammatory mediators involved in SAR, nasal lavage fluid (NLF) from SAR patients has previously been analysed with regard to a variety of well-known proinflammatory compounds such as histamine,⁸ arachidonic acid metabolites,⁹ platelet-activating factor,¹⁰ cytokines,^{8,11} chemokines,¹² and neurotransmitters.¹³ By contrast, no proteomic study of NLF from SAR patients has been reported. We have earlier described the protein pattern of human NLF analyzed with 2-DE and identified a large number of proteins that are implicated in airway inflammatory and immune responses.^{14–16} Furthermore, changes in the NLF 2-DE protein pattern have been demonstrated in individuals with occupational asthma,¹⁷ upper airway irritation¹⁸ and sinusitis,¹⁹ indicating that analysis of the NLF proteome may reveal new features of human airway disease.

In the present investigation, we have compared the NLF 2-DE protein patterns of SAR patients and healthy controls. NLF samples were taken in both groups both before allergy (pollen) season and during season, and twenty proteins in the

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2-DE pattern were identified by mass spectrometry and quantified by a computerized imaging system. Many of the examined proteins are either known or suspected to take part in inflammatory reactions, e.g., the endogenous proteinase inhibitors, cystatin S and von Ebner's gland protein,^{20–21} and the newly discovered lipopolysaccharide-binding protein, PLUNC (palate lung nasal epithelial clone).^{22–23} One previously unidentified NLF protein was found in all samples from the SAR patients during allergy season but not in any sample before season: this protein was identified as eosinophil lysophospholipase (Charcot-Leyden crystal protein/galactin 10) and was shown to be acetylated at the N-terminus. The possibility that this NLF protein may be a new marker of SAR is inferred.

Material and Methods

Chemicals. Iodoacetamide, DTT, sodium dodecyl sulfate (SDS), CHAPS, α -cyano-4-hydroxycinnamic acid, trifluoroacetic acid (TFA), sodium azide, potassium ferricyanide and dihydrobenzoic acid (DHB) were acquired from Sigma (Steinheim, Germany). TEMED, 40% acrylamide solution, 2% bis acrylamide solution and ammonium persulfate were purchased from Bio-Rad (Hercules, CA). Urea (pro analysis) was from Fluka (Buchs, Switzerland), and acetonitrile and acetic acid from Riedel-de Haën (Seelze, Germany). IPGs NL pH 3–10, IPG buffer pH 3–10 NL and dry strip cover fluid were purchased from Amersham Bioscience (Uppsala, Sweden). Porcine trypsin was from Promega (Madison, WI). The calibration mixture for peptide mass fingerprinting, des-Arg1-bradykinin, angiotensin 1, Glu1-fibrinopeptide B, neurotensin, adrenocorticotrophic hormone (ACTH, clip 1–17), ACTH (clip 18–39), ACTH (clip 7–38) with masses: 904.4681, 1296.6853, 1570.6774, 1672.9175, 2093.0867, 2465.1989, 3657.9294, respectively, was purchased from Applied Biosystems (Foster City, CA).

Subjects. Eleven subjects volunteered for the investigation: six patients with seasonal allergic rhinitis (SAR) due to birch (2 male, 2 female) and grass pollen allergy (2 female) and five healthy subjects (2 male, 3 female) serving as controls. The inclusion criteria were as follows: (i) a skin prick test verifying their pollen allergy but without signs of sensitization to any perennial allergen in the SAR group and no signs of sensitization to any inhalant allergen in the control group; (ii) no history of upper airway infection three weeks prior to examination and no signs of infection at anterior rhinoscopy; (iii) nonsmoking habits. The skin prick test included inhalant allergen extracts: birch pollen, timothy pollen, pollen from *Artemisia vulgaris*, cat-, dog-, and horse dander, mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), and molds (*Alternaria*, *Cladosporium*) from ALK, Denmark. All eleven subjects were examined by anterior rhinoscopy and nasal lavage at two different occasions; (i) before pollen season (in February), and (ii) during pollen season (in April). Thus, the examination confirmed ongoing SAR in the patient group during pollen season, but did not detect any signs of inflammation or irritation before nasal sampling at either occasion in the control group or out of pollen season in the SAR group. All subjects scored their rhinitis symptoms at each sampling occasion. Four different rhinitis symptoms (itching, sneezing, secretion, and stuffy nose) were scored from 0 (= no symptom) to 6 (= extremely troubled); these were then added together to give a combined nasal symptom score. All individuals gave their consent to participate after full information. The study was approved by the Ethics Committee at the University Hospital of Linköping Sweden (Dnr 00–056).

Samples. NLFs were obtained from 15-mL saline washings of the nasal mucosa as described elsewhere.¹⁴ The samples were centrifuged to remove cellular debris and the total protein concentration was determined with Bio-Rad (Richmond, CA) protein assay according to Bradford.²⁴ A portion (2.5 mL) of the sample was then desalted, lyophilized and dissolved in urea sample solution according to Görg et al.²⁵

Two-Dimensional Gel Electrophoresis (2-DE) Analysis. 2-DE was performed in a horizontal 2-DE set up (IPGphor and Multiphor from Amersham Bioscience) as described in detail previously¹⁴ and essentially according to Görg.²⁵ One hundred μ g protein per sample was applied on IPGs NL pH 3–10 by in-gel rehydration (according to manufacturer's instructions). Separation of proteins in the first dimension was followed by two equilibration steps of the IPGs in SDS equilibration buffer, after which proteins were transferred to gradient SDS-PAGE gels cast on GelBond PAG film ($0.5 \times 180 \times 245$ mm, 11–18% T, 1.5% C, 33–0% glycerol) running at 20–30 mA for about 6 h.

Staining and Image Analysis. The separated proteins were detected by fluorescent staining (SYPRO Ruby, Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's staining protocol (SYPRO Ruby protein gel stain web site: www.probes.com), that is; after SDS-PAGE, gels were fixed using 10% methanol/7% acetic acid solution for 30 min and then incubated in 400 mL SYPRO Ruby protein gel stain solution overnight. Gels were finally washed and placed in deionized water at 4 °C. All staining and washing steps were performed with continuous gentle agitation. The gels were also stained with silver according to Shevchenko²⁶ as described previously²³ after SYPRO Ruby staining. These gels were incubated in 10% glycerol overnight and then covered with a moist cellophane sheet and air-dried.

The 2-DE protein patterns were visualized and analysed using a CCD (Charged-Coupled Device) camera digitizing at 1340×1040 pixels resolution, for SYPRO Ruby gels using an UV-scanning illumination mode (Fluor-S Multi-Imager from Bio-Rad, Hercules, CA) in combination with a computerized imaging 12-bit system designed for evaluation of 2-DE patterns (PDQuest version 7.1.0, Bio-Rad). By placing the gel with the polyacrylamide side down, background fluorescence from the plastic bond was eliminated. The different images were evaluated using an approach as described previously.¹⁶ In short, the gel image with most detected protein spots was used as a master gel and all the other images were then matched to the master gel. To correct for differences in total staining intensity between different images, the volume of the fluorescent intensity of each spot was divided by the total volume of all spots in the same gel, multiplied by a scaling factor of 100. Thereby % values for all proteins were generated that were evaluated for significant differences between the groups.

Enzymatic Digestion. The protein spots were visualized and excised on a blue light transilluminator (Dark ReaderTransilluminator DR-180B from Clara Chemical Research, Denver, CO) in a dark room using amber glasses provided with the instrument. The protein spots were excised from the gel manually with a homemade spotpicker, which consisted of a syringe coupled to a 2 mm diameter Teflon-coated metal head, and transferred to small-siliconized Eppendorf tubes (0.5 mL). Proteins were cleaved by trypsin into peptides as described previously.¹⁶ Silver stained gel pieces were destained with 15 mM potassium ferricyanide/50 mM sodium thiosulfate as described previously,²³ before tryptic digestion.

Enzymatic Release of Oligosaccharides. To examine glycosylation, NLF proteins were treated with an *N*-glycosidase, PNGase F (Sigma, Steinheim, Germany), by using the condition recommended by the supplier. NLF was desalted, lyophilized and dissolved in 200 μ L urea sample buffer. The sample (100 μ L) containing about 600 μ g protein was incubated at 37 °C overnight in the presence and absence of 40 μ L PNGase (500 units/mL). The reaction was stopped and the PNGase-treated sample and the control were analyzed by 2-DE. The gels were stained by Coomassie brilliant blue and the two protein patterns compared.

To examine sialylation, NLF proteins were digested by 10 mUnit α - (2 $\rightarrow$ 3, 6, 8, 9) neuraminidase (Sigma, Steinheim, Germany) following manufacturer's instructions. After incubation at 37 °C for 3 h, the proteins were desalted, lyophilized and dissolved in urea sample solution. The neuraminidase-digested sample and a control sample that was treated by the same procedure but in the absence of the enzyme, were stored at -20 °C until analyzed by 2-DE.

Peptide Mass Fingerprinting and Tandem Mass Spectrometry. The peptides obtained after tryptic digestion were mixed with the matrix and applied directly onto the MALDI plate.¹⁶ Analysis of peptide masses were performed using MALDI-TOF MS (Voyager-DE PRO, Applied Biosystems, CA, USA) equipped with a 337 nm laser and delayed extraction and operated in reflector mode. Laser intensity was set to 1800, positive ionization mode, and a delay time of 170 ns was used. Spectra in the mass range between 600 and 3600 Da were collected from about 300 shots. Data processing of the spectra were performed with Data Explorer version 4.0 (Applied Biosystems, Foster City, CA) and close external mass calibration with a standard peptide mixture was used. The spectra were also internally calibrated using known trypsin autolysis peaks (m/z : 842.5200, 2211.1046).

For nanoelectrospray MS/MS analysis the remaining tryptic digested peptides were dried and dissolved in 10 μ L 0.1% TFA.²³ The peptides were desalted, cleaned up by using Millipore Zip Tip_{C18} column, applied into a silver coated glass capillary, and analyzed by a hybrid mass spectrometer API Q-STAR Pulzer i (Applied Biosystems, Foster City, CA) equipped with nanoelectrospray ion source (MDS-Protana, Odense, Denmark) operated in nanopositve mode. Data processing were performed with Analyst QS software (Applied Biosystems, Foster City, CA). Fragmentation spectra were interpreted manually.

Database Searches. The mass list (mass + H^+) generated from the major peaks of the MALDI spectra was submitted to a database search (NCBI or SWISS-PROT) using PeptIdent and MS-FIT search engines. Restrictions were placed on species (Human), mass tolerance (± 25 ppm), maximum missed cleavages by trypsin (up to 1) and cysteine modification by carbamidomethylation. The resulting tandem mass spectra were used for peptide sequence determination and the derived tags were searched against NCBI database using MS-Pattern search engine.

Statistical Analysis. Values are given as mean $\pm$ standard deviation (SD). The significance of differences ($p < 0.05$) were calculated using a nonparametric test for unpaired observations, Wilcoxon's rank sum test (Mann Whitney U-test). The data set generated from 2-DE analysis was also analyzed by hierarchical cluster analysis using the Ludesi 2-DE interpreter software package available at www.ludesi.com. The quantitative data derived from PDQuest was exported to the Ludesi 2-DE interpreter. The clustering procedure includes the generation

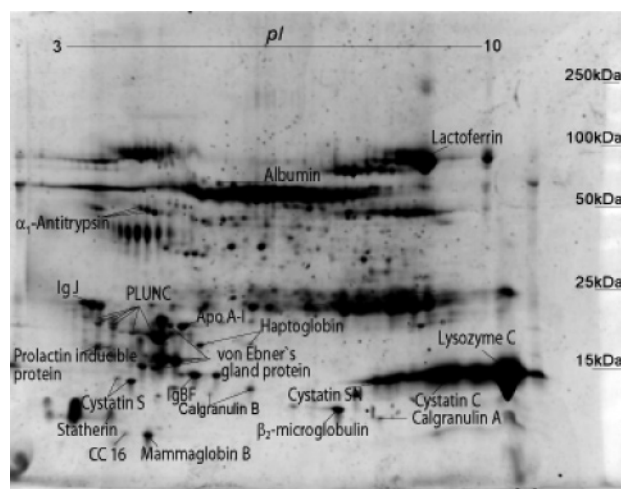


Figure 1. 2-D gel electrophoregram obtained from a healthy human subject. Proteins (100 μ g) were first separated on p/3–10 nonlinear IPG strips (first dimension) and then on 11–18%T SDS-polyacrylamide gels (second dimension). The gel was stained with SYPRO Ruby, which has a detection limit of about 10 ng protein. Proteins analyzed in the present study are indicated with their names.

of a distance matrix, which in our case was calculated by the Pearson correlation distance on the 50 proteins with lowest *p*-value selected from an ANOVA (Analysis of Variance) procedure, summarizing all the pairwise similarities between expression profiles and generating a dendrogram (hierarchical tree). The clustering was based on a significant number of proteins which appear coexpressed within the different subject groups (SAR patients or controls) and at different time points (during or before pollen season).

Results

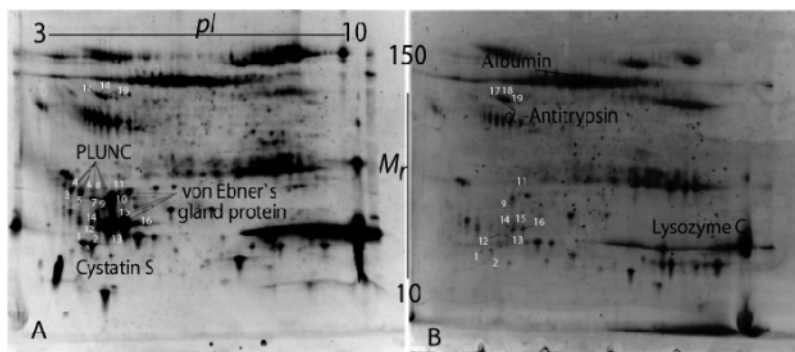
Nasal Symptom Scores. The nasal symptom score during pollen season was significantly higher in SAR patients than controls (10.5 ± 2.0 vs. 0.0 ± 0.0 , $p = 0.004$), whereas there was no difference between the groups when examined before pollen season (1.0 ± 0.5 vs. 0.2 ± 0.2 , $p > 0.05$). Consistently, the score was significantly higher during pollen season than out of season in SAR patients (10.5 ± 2.0 vs. 1.0 ± 0.5 , $p = 0.03$) but not in controls (0.0 ± 0.0 vs. 0.2 ± 0.2 , $p > 0.05$). Samples obtained during pollen season were taken early in the season shortly after onset of upper airway symptoms in SAR patients. Pollen counts in the area indicated high birch pollen levels (> 1000 p/m³) during these days.

Nasal Fluid 2-DE Protein Pattern. Figure 1 show a typical NLF 2-DE protein pattern obtained from a healthy control subject. Although an impressive number of proteins are detected in NLF by the 2-DE technique it should be noted that the protein patterns are even more complex since very low abundant proteins (e.g., cytokines), proteins with very high and low molecular masses and proteins with extreme isoelectrical points (e.g., > 10) are not readily detected on 2-D gels. Analysis of the gel by PDQuest revealed about 800 spots that could be resolved. Immunoglobulins, such as IgA, are abundant proteins in NLF protein patterns,¹⁴ but we have previously also identified a large number of other NLF proteins,¹⁶ many of which are implicated in airway inflammatory and immune responses. To assess overall differences in NLF 2-DE pattern between different groups, we applied a hierarchical cluster analysis.²⁷ In the

Table 1. Protein Changes in Human NLF from SAR Patients and Controls before and during Pollen Season. Spot Numbers Refer to Marked Spots in Figure 2^a

protein	spot no.	M_r/pI	before allergy season		during allergy season	
			controls	rhinitis	controls	rhinitis
Cystatin S	1	13.5/4.9	0.68 ± 0.25	0.72 ± 0.56	0.50 ± 0.33	0.26 ± 0.18^b
PLUNC	2	13/5.1	0.12 ± 0.08	0.17 ± 0.16	0.06 ± 0.05	0.08 ± 0.18
	total	0.37 ± 0.34	0.25 ± 0.30	0.42 ± 0.40	0.06 ± 0.30^b	0.06 ± 0.30^b
von Ebner's gland protein	3	27/5.1	0.05 ± 0.05	0.05 ± 0.08	0.04 ± 0.05	0.00 ± 0.00
	4	27.5/5.1	0.07 ± 0.08	0.00 ± 0.00 ^b	0.06 ± 0.06	0.00 ± 0.00 ^b
	5	26/5.2	0.05 ± 0.05	0.07 ± 0.08	0.08 ± 0.08	0.02 ± 0.04
	6	27/5.2	0.05 ± 0.05	0.05 ± 0.08	0.10 ± 0.07	0.00 ± 0.00 ^b
	7	25/5.3	0.08 ± 0.08	0.05 ± 0.05	0.08 ± 0.08	0.02 ± 0.04
	8	26/5.3	0.07 ± 0.05	0.03 ± 0.05	0.06 ± 0.09	0.02 ± 0.04
	total	4.22 ± 1.42	2.87 ± 2.54	3.80 ± 1.96	1.40 ± 1.21^b	1.40 ± 1.21^b
α_1 -Antitrypsin	11	27.5/5.4	0.70 ± 0.38	0.37 ± 0.29	0.78 ± 0.48	0.22 ± 0.24 ^b
	12	17/5.2	0.30 ± 0.11	0.20 ± 0.23	0.26 ± 0.18	0.18 ± 0.30
	13	17/5.3	0.38 ± 0.08	0.48 ± 0.48	0.34 ± 0.29	0.12 ± 0.13 ^b
	14	17.5/5.4	0.52 ± 0.51	0.17 ± 0.19	0.26 ± 0.23	0.08 ± 0.08 ^b
	15	18/5.4	0.78 ± 1.12	0.43 ± 0.50	0.78 ± 0.33	0.26 ± 0.42
	16	17.5/5.5	1.53 ± 0.47	1.22 ± 1.21	1.38 ± 0.70	0.54 ± 0.53 ^b
	total	0.30 ± 0.26	0.33 ± 0.09	0.28 ± 0.22	0.50 ± 0.35	0.50 ± 0.35
Eosinophil lysophospholipase	17	58/5.2	0.05 ± 0.05	0.10 ± 0.00 ^b	0.04 ± 0.05	0.14 ± 0.09 ^b
	18	57/5.3	0.13 ± 0.12	0.12 ± 0.04	0.14 ± 0.09	0.22 ± 0.18
	19	56/5.4	0.12 ± 0.10	0.12 ± 0.04	0.10 ± 0.10	0.14 ± 0.13
		14.5/6.1	0.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.39	0.78 ± 0.48 ^b

^a The protein levels were measured as fluorescence intensity (counts) and are expressed as percentage of total protein stain intensity. The values are mean $\pm$ SD. ^b Significant difference $p < 0.05$ compared to controls.

**Figure 2.** 2-D images of NLF proteins in a SAR patient before (A) and during (B) pollen season. Proteins were analyzed as described in the legend to Figure 1. The proteins whose levels were changed during pollen season are designated with their names. The marked spot numbers refer to the protein isoforms in Table 1.

resulting tree diagram (dendrogram), the patient samples formed two clusters; those taken before vs those taken during pollen season, on the basis of similarity of the protein patterns within each group ($p < 0.05$, ANOVA).

Protein Analysis. To investigate the possibility that specific NLF proteins in the 2-DE pattern might be markers of allergic rhinitis, we selected 20 proteins that were well represented in all subjects at both sampling occasions (Figure 1). The identities of the proteins were confirmed by peptide mass fingerprinting, showing 20–81% sequence coverage. Several of these proteins are known to take part in inflammatory reactions and suggested to be involved in the pathogenesis and/or pathophysiology of rhinitis.^{28–29} Most of the examined proteins were in the 10–30 kDa molecular range (Figure 1) but three larger proteins (α_1 -antitrypsin 56–58 kDa), albumin (66–69 kDa), and lactoferrin (80 kDa) were also included in the study.

Out of the 20 proteins, 5 showed different levels in SAR patients as compared to controls during pollen season (Table 1). Thus, the levels of six acidic isoforms of PLUNC (M_r/pI : 27/

5.1, 26/5.2, 25/5.3, 27.5/5.1, 27/5.2 and 26/5.3) were lower in SAR patients (Figure 2, spot no. 3–8), as were the levels of six isoforms of von Ebner's gland protein (Figure 2, spot no. 11–16) and of M_r/pI : 13/5.1 cystatin S (Figure 2, spot no. 2). On the other hand, the level of M_r/pI : 58/5.2 α_1 -antitrypsin (Figure 2, spot no. 17) was higher in patients than controls. Before allergy season, there were no statistical differences between SAR patients and controls regarding total PLUNC, VEGP, or cystatin S, while the levels of the M_r/pI : 58/5.2 isoform of α_1 -antitrypsin were higher in patients than controls also at that time (Table 1).

Figure 2 show typical electrophoregrams obtained from a SAR patient before and during pollen season. As indicated, the levels of PLUNC, VEGP, and cystatin S were lower during season than before season, while the level of α_1 -antitrypsin (M_r/pI : 58/5.2) was higher. Notably, the levels of albumin and lysozyme were significantly higher during pollen season than before season in both patients and controls. Quantitative determinations showed that both groups had about 2-fold

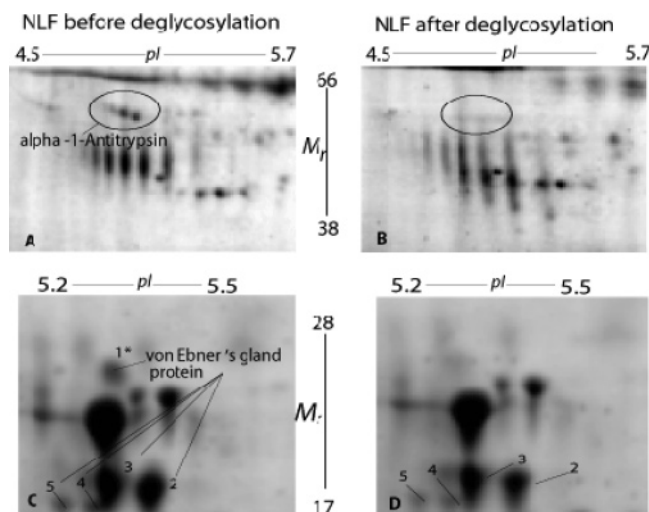


Figure 3. Deglycosylation of proteins in human NLF. Nasal fluid proteins were incubated in the absence (A and C) and presence (B and D) of PNGase F. The proteins were then separated with 2-DE and stained with Coomassie brilliant blue. The disappearance of α_1 -antitrypsin after removal of carbohydrates is marked with an ellipse in B. The five marked protein spots in (C) represent isoforms of von Ebner's gland protein that are normally expressed in NLF. The 1* indicates the isoform that is N-glycosylated and disappeared after PNGase treatment (D).

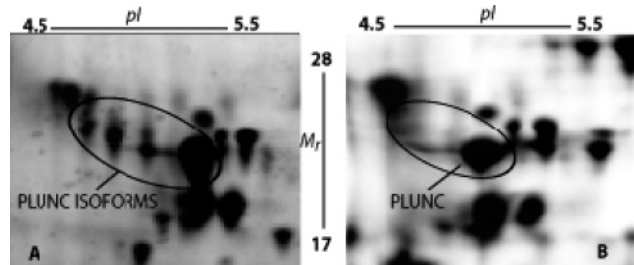


Figure 4. Neuraminidase digestion of proteins in human NLF. (A) Typical 2-DE pattern of PLUNC isoforms in NLF. (B) Desialylation resulted in a decrease of all acidic isoforms.

higher levels of albumin and lysozyme in NLF during pollen season than before.

Glycosylation. When NLF proteins were subjected to digestion with PNGase, the 2D positions of several known glycosylated proteins such as α_1 -antitrypsin (Figure 3A and B) were dramatically changed compared to the control sample. Notably, the VEGP isoform at position M_r/pI : 27.5/5.4 (Figure 3C) disappeared after PNGase treatment (Figure 3D). The electrophoretic mobility shift assay with neuraminidase showed no changes in the position of VEGP but the position of all PLUNC isoforms were changed (Figure 4A and B). As shown in Figure 4B, PLUNC was detected as only one spot (at M_r/pI : 24.8/5.4) after neuraminidase treatment.

Identification of Eosinophil Lysophospholipase. One previously unidentified protein (M_r/pI : 14.5/6.1) was found in NLF from SAR patients during allergy season (Figure 5B) but not before season (Figure 5A). To identify this protein, the spot was picked, destained, trypsinized and analyzed by MALDI-TOF MS (Figure 6). Nine peptides that cover 73.8% of the protein (Table 2) identified it as eosinophil lysophospholipase (ELP, Swiss-Prot accession no. Q05315). After silver staining (detection limit 1–10 ng), the protein was found in NLF from all SAR patients

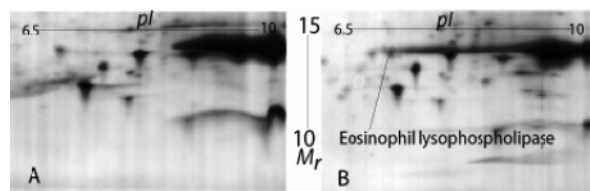


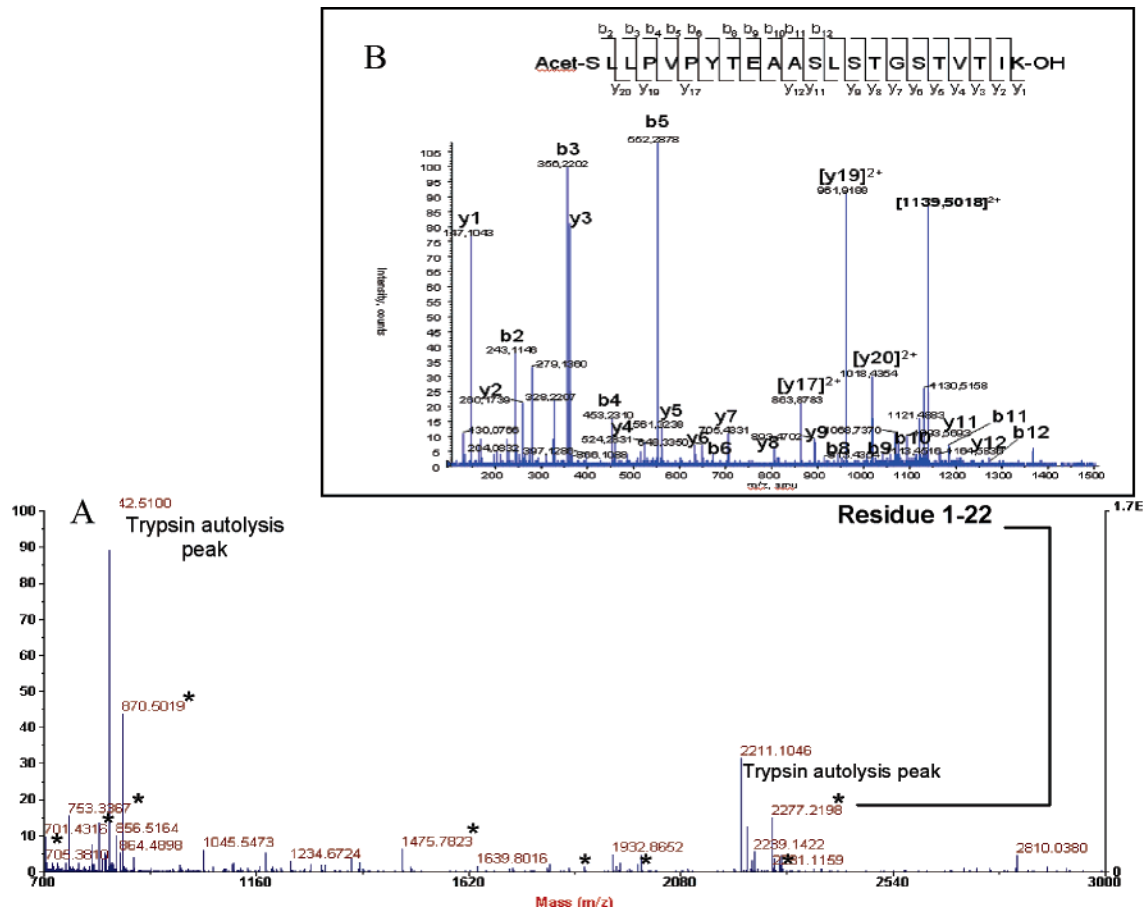
Figure 5. Partial NLF 2-DE protein pattern in a SAR patient before (A) and during pollen season (B). Proteins were separated as described in the legend to Figure 1 and stained with silver. The protein spot at M_r/pI : 14.5/6.1 was cut out, destained, trypsinized and identified by mass spectrometry as eosinophil lysophospholipase (cf Figure 6).

and two of the controls during pollen season, but was not detected in any patient or control before pollen season (Table 1). The identity as ELP was verified in three peptides by amino acid sequencing after analysis of collision-induced (CID) fragmentation spectra with nanoelectrospray MS/MS (Table 2). The N-terminal sequence of ELP begins with Met, but the corresponding peptide ion did not appear in the MS spectra. Instead, we found a peptide ion with a molecular mass 42 Dalton higher than the predicted mass for the peptide, indicating the removal of Met and acetylation of the Ser (residues 1–22, Acet-SLLPVYTEAASLSTGSTVTIK–OH, m/z : 2277.2224). As shown in Figure 6B, the acetylation of the N-terminus of the protein was verified by nanoelectrospray MS/MS of the doubly charged peptide. The most intense ions corresponding to sequential cleavages of the $[MH]^{+2}$ ion of ELP were readily identified and almost a complete series of y-type and b-type fragment ions were present.

Discussion

The object of this investigation was to compare the NLF proteome during nasal inflammation with that during normal noninflammatory conditions. It was essential, therefore, to ascertain that the SAR patients had ongoing nasal inflammation during pollen season (but not out of season) and that the controls had no inflammation. Accordingly, both the symptom score and the visual examination verified an inflammation in the SAR patients during pollen season, while there were no symptoms or signs of inflammation before pollen season. Controls showed no signs of inflammation or irritation at either occasion.

The results indicate that, during allergy season, the levels of PLUNC in NLF were lower in SAR patients than controls, as were the levels of VEGP and of cystatin S. By contrast, the levels of α_1 -antitrypsin with M_r/pI : 58/5.2 was higher in SAR patients than controls. PLUNC is a recently identified protein in human NLF^{22–23,30} whose gene expression is restricted to the upper airways.³¹ Its precise function(s) has not yet been determined, but it has sequence homology with well-known antibacterial proteins such as LBP (lipopolysaccharide-binding protein) and BPI (bactericidal/permeability-increasing protein) and is suggested to act in the host defense³² and to protect against infections after epithelial injury.³³ Accordingly, experiments in vitro have demonstrated that PLUNC is the only protein in human NLF that binds to LPS.²³ We have previously identified 8 isoforms of PLUNC in NLF (No. 3–10 in Figure 2) with pI s between 5.1 and 5.5 and molecular masses between 25 and 27.5 kDa²³ and reported decreased levels of PLUNC in NLF from smokers¹⁶ and subjects occupationally exposed to chemical irritants.³⁰ In the present investigation, we demonstrate that



decreased in NLF of SAR patients is consistent with its tentative role as a cysteine proteinase inhibitor. Raftery et. al.³⁶ have studied grass pollen and found that some pollen allergens have proteinase activities that are able to damage the airway epithelium, and this has been confirmed by other studies.^{37–38} If released proteinases from the inhaled pollen are not inhibited by the endogenous proteinase inhibitors they can degrade the antiproteinases and continue to damage the airway epithelial cells.³⁹ Furthermore, cystein-proteases from dust mites induce proinflammatory cytokines through activation of protease activated receptor 2.⁴⁰ Cystatins and other proteinase inhibitors such as VEGP are therefore important to maintain a healthy state. In this context, it may be noted that asthmatic patients have a distorted proteinase/antiproteinase balance.⁴¹ Along this line, it may be hypothesized that the decreased levels of cysteine proteinase inhibitors, VEGP and cystatin S, may contribute to the tissue injury and inflammation in the nasal mucosa of SAR patients.

As opposed to the levels of PLUNC, VEGP, and cystatin S, the levels of α_1 -antitrypsin were higher in SAR patients than controls. α_1 -Antitrypsin is a serine proteinase inhibitor that inactivates proteolytic enzymes such as elastase, thus forming a defense mechanism against elastase-mediated tissue injury. In addition, α_1 -antitrypsin was recently shown to inhibit IgE-dependent histamine release from human mast cells,⁴² suggesting a protective role in allergy. As shown in Figure 2, α_1 -antitrypsin is found as three isoforms (M_r/pI : 56–58/5.2–5.4) in the human NLF 2-DE protein pattern. All three appear glycosylated (Figure 3A and B), which agrees with the reported finding of several N-linked glycosylated forms of α_1 -antitrypsin in human plasma.⁴³ As to which particular isoform(s) was increased, the increment in the most glycosylated form (M_r/pI : 58/5.2) reached statistical significance while the others did not. It is possible that this reflects an increased posttranslational glycosylation of α_1 -antitrypsin in SAR, but further studies must be undertaken to test this hypothesis.

There were no statistical differences between SAR patients and controls regarding total PLUNC, VEGP, or cystatin S before allergy season, while the levels of M_r/pI : 58/5.2 α_1 -antitrypsin were higher in SAR patients than controls also at that time. The finding that the M_r/pI : 58/5.2 α_1 -antitrypsin was higher in SAR patients also before pollen season, i.e., at a time when there were no signs or symptoms of nasal inflammation, suggests that other factors than inflammatory mediators may be responsible. Further studies must be undertaken to examine whether there are genetic or other factors in SAR patients that may explain the high levels of M_r/pI : 58/5.2 α_1 -antitrypsin in the nasal fluid.

The present study identified, for the first time, eosinophil lysophospholipase (ELP) in human NLF. This protein (Swiss-Prot accession no. Q05315) is abundantly expressed by eosinophils and can therefore be regarded as a hallmark of eosinophil leukocyte infiltration associated with allergic disease. Accordingly, ELP was found in NLF from all SAR patients during pollen season, but not in any patient before pollen season. Previous studies have demonstrated that ELP is localized in the primary granules of eosinophils, from where it may be secreted or transported to other locations.⁴⁴ Our findings indicate that, during pollen season, ELP is released from mucosal eosinophils and secreted into the nasal fluid. Miike et. al.⁴⁵ have suggested a mechanism in which cysteine proteinases derived from allergens activate eosinophils and induce the release of granule proteins. Our findings are consistent with such a mechanism,

not least because the NLF levels of the cysteine proteinase inhibitors, cystatin S and VEGP, were decreased in SAR patients during the pollen season and so might have failed to inhibit the proteinase action. Further studies are required to explore the finding that ELP was found in NLF of two control subjects at pollen season. It is possible that pollen constituents may cause a minor degranulation of nasal eosinophils also in healthy subjects, but that SAR patients have a much more profound mucosal eosinophil infiltration and/or degranulation.⁴⁶

Nanoelectrospray MS/MS of the N-terminus of the ELP showed removal of Met and acetylation of Ser. Acetylation of Ser² and N-linked glycosylation of Asn¹³⁵ has previously been predicted in ELP.⁴⁷ Our results confirm the acetylation but not the glycosylation as the unmodified corresponding peptide, position 134–140, was sequenced (Table 2). Cleavage of N-terminal Met and acetylation of Ser are common co- and post-translational modifications of proteins that may affect the activity and stability of the protein.⁴⁸ In this case, the biological implication of N-terminal acetylation of ELP in human nasal fluid remains to be clarified. Hypothetically, it may affect the enzyme activity and so the capacity to degrade inflammatory-promoting lysophospholipids. Notably, Chilton et. al.⁴⁹ has found high levels of lysophospholipids in antigen-stimulated airways and lysophosphatidylcholine is thought to be involved in the pathophysiology of bronchial asthma.⁵⁰

As to the other proteins examined (Figure 1), there were no significant differences in NLF levels between SAR patients and controls. This speaks against a major role for these proteins in SAR. It should be emphasized, however, that a lack of difference in overall protein levels does not preclude differences in biological or enzyme activity, and further studies taking these considerations into account must be undertaken to clarify the role of different proteins in SAR. The mean level of Clara cell protein (CC16) was lower in SAR patients than controls during pollen season, but there was great variation between individuals and the *p* value for difference between the groups did not reach statistical significance. Decreased CC16 levels have been demonstrated in NLF of children with allergic rhinitis.^{29,51} The discrepancy compared to this study may reflect differences in adults versus children⁵² but, a larger study of a homogeneous group of SAR patients is warranted.

The levels of albumin and lysozyme were significantly increased during pollen season in both patients and controls. Thus, there were about 2-fold higher levels of albumin and lysozyme in NLF during pollen season than before. Albumin is a well-known marker of increased vascular permeability⁵³ and it is therefore possible that pollen-induced plasma exudation could explain the higher level of albumin in subjects during the pollen season. Lysozyme is an antimicrobial protein whose release from mucosal glands is increased after cholinergic challenge.^{28,54}

In conclusion, we have obtained evidence to indicate that the levels of several proteins (PLUNC, VEGP, cystatin S, α_1 -antitrypsin, and eosinophil lysophospholipase) are significantly changed in the NLF of SAR patients during nasal inflammation. In addition, our findings suggest the possibility that the M_r/pI : 58/5.2 α_1 -antitrypsin may be higher in NLF of SAR patients also when there are no signs or symptoms of nasal inflammation. Altogether, the findings illustrate the potential use of proteomics for identifying new markers of allergic inflammation and for revealing structural details of such markers. In particular, the data highlight the importance of exploring isoform-specific

levels of proteins in patho-physiological conditions since modifications of proteins determine their activity state, localization, turnover, and interaction with other molecules. From the clinical point of view, the present work should be regarded as a pilot study. An investigation with a larger number of subjects is necessary to establish if these findings apply to the majority of SAR patients.

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