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Nasal lavage fluid and proteomics as means to identify the effects of the irritating epoxy chemical dimethylbenzylamine

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The aims of this study were to describe the changes in the nasal lavage fluid (NLF) protein pattern after exposure to the irritating epoxy chemical dimethylbenzylamine (DMBA) and to identify the affected proteins using a proteomic approach. The protein patterns of NLF from six healthy subjects and eight epoxy workers with airway irritation were analysed using two-dimensional gel electrophoresis (2-DE) before and after exposure to 100 μg m^{-3} DMBA for 2 h in an exposure chamber. NLF proteins were identified by (i) comparison with a 2-DE NLF reference database; (ii) N-terminal amino acid sequencing; and (iii) mass spectrometry. In NLF from healthy subjects, the levels of immunoglobulin A increased and the levels of Clara cell protein 16 (CC16) decreased after chamber exposure, while in NLF from epoxy workers, α_2-macroglobulin and caeruloplasmin increased. Two previously unidentified proteins decreased in NLF from epoxy workers after exposure; these were identified as statherin and calgranulin B. In addition, the subjects who developed high counts of eosinophils in their nasal mucosa after chamber exposure had significantly lower levels of immunoglobulin-binding factor (IgBF) before exposure than subjects with low eosinophil infiltration. These results show that short-term exposure to DMBA causes distinct changes in NLF proteins. Moreover, three proteins that have previously not been associated with upper airway irritation were identified: statherin, calgranulin B and IgBF. Further studies are needed to investigate whether these proteins may be used as biomarkers of airway irritation and to give new insight into the ways in which occupational exposure to irritants causes inflammation of the airways.

Keywords: nasal lavage fluid, dimethylbenzylamine, epoxy, mass spectrometry, proteomics.

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Introduction

The incidence of occupational rhinitis and asthma is increasing, and irritation of the upper airways is a major problem in many work places. Therefore, there is a need for markers of airway irritation. Moreover, because rhinitis appears to be a risk factor for asthma (Vignola *et al.* 1998, Passalacqua *et al.* 2000), the identification of proteins that indicate upper airway irritation may be highly relevant to the detection of individuals at an early stage of the disease. Nasal lavage is an invasive and simple technique for collecting samples from the upper airways and is therefore suitable for use in the search for such markers. Although nasal lavage fluid (NLF) has been examined in several studies of environmental exposures, very few of these studies include exposure to chemical irritants (Norbäck and Wieslander 2002). NLF

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contains thousands of proteins that altogether possess great potential to serve as markers of airway irritation and to give new insights into the mechanisms behind inflammatory diseases of the respiratory tract. Two-dimensional gel electrophoresis (2-DE), which separates proteins according to charge and size and leads to a protein spot pattern (Lindahl *et al.* 1998), can be used to examine these proteins. This technique has been shown to have the highest resolution for protein analysis and is now a cornerstone in the new field of proteomics, that is linking the proteome with the genome. In combination with new technologies such as microsequencing and peptide mass fingerprinting with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), it has become easier to identify the proteins separated by 2-DE and this has opened up new possibilities to gain detailed information about disease processes (Hochstrasser 1998, Banks *et al.* 2000). Proteomics have been applied in the field of toxicology (Steiner and Anderson 2000), and have great potential both in investigative studies and in screening/predictive toxicology (Kennedy 2002). Previous investigations have also demonstrated that 2-DE may be used to detect changes in NLF and bronchoalveolar lavage fluid (BALF) protein patterns in subjects with airway diseases such as pulmonary fibrosis, asthma or pleuritis (Lindahl *et al.* 1995, *1996*, Wattiez *et al.* 2000). The NLF 2-DE patterns contain many proteins of potential clinical interest, including lipocortin-1, Clara cell protein 16 (CC16), lipocalin-1, cystatin S and immunoglobulin binding factor (IgBF) (Lindahl *et al.* 1999a, b).

By using MALDI-TOF MS, we recently identified a new protein, palate lung nasal epithelial clone (PLUNC), and demonstrated that its levels in NLF were changed after exposure to the irritating epoxy chemical dimethylbenzylamine (DMBA) (Lindahl *et al.* 2001). Furthermore, we previously reported that short-term exposure to low air concentrations of DMBA caused infiltration of eosinophils in the nasal mucosa of healthy individuals (Irander *et al.* 1997). It is important, therefore, to study in more detail the changes that occur in the airways during exposure to DMBA, and to find markers of airway irritation caused by reactive chemicals in occupational settings such as the epoxy industry.

In this study, we use a proteomic approach to demonstrate other changes in the 2-DE NLF protein patterns of subjects exposed to low levels of DMBA and to identify previously unknown proteins whose levels in NLF are affected by such exposure.

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Materials and methods

Subjects

NLF and mucosal cells were collected from six healthy subjects (non-smokers, median age 34 years, range 31–38 years) and from eight epoxy workers (six smokers and two non-smokers, median age 37 years, range 26–55 years) with occupational exposure to DMBA. Samples were taken before and after 2 h' exposure to DMBA in an exposure chamber (see below). For comparison, samples were also collected from seven healthy smokers (median age 48 years, range 24–55 years) without any known exposure to DMBA. All subjects were examined clinically with anterior rhinoscopy, and showed no mucosal changes before or after exposure.

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Occupational history

The workers who participated in this study worked in a plant producing electrical capacitors. The median time of employment was 4.5 years (range 3–19 years). The production process involved a heat-cured epoxy system using hexahydrophthalic anhydride (HHPA) (30%) as the hardener and DMBA (0.3%) as the accelerator. Most work was performed in closed systems or ventilated hoods, but some operations included handling of uncured HHPA mixtures at elevated temperatures. The subjects complained of work-related symptoms in the upper airways, e.g. secretion, blockage and sneezing (Nielsen *et al.* 1994). Air levels of 3–470 μg m^{-3} HHPA during an 8 h work-shift (Welinder *et al.* 1994) and up to 100 μg m^{-3} of DMBA during a 2 h work-shift were recorded.

Exposure chamber

Gaseous DMBA was generated into an exposure chamber as described previously (Ståhlbom *et al.* 1997). The experiments were carried out for 2 h at 100 μg m^{-3}, and the coefficient variation of time weighted average (TWA) air concentrations of DMBA were 2–6%. Six of the eight epoxy workers developed upper airways symptoms such as nose blockage, secretion and sneezing during exposure. The other two developed lower airways symptoms such as dry cough, hacking cough and smarting pain. One subject experienced eye irritation. The controls did not experience an increase in symptoms after chamber exposure (Irander *et al.* 1997).

Samples

Nasal lavages, nasal brushings and rhinoscopy were carried out with the informed consent of the subjects and as approved by the ethical committee of the Faculty of Health Sciences, University of Linköping, Sweden. NLF was obtained by saline washings of the nasal mucosa performed by means of a modified 'nasal pool' device (Greiff *et al.* 1990). For this, 15 ml of saline was inserted via a syringe and a Foley catheter into the left nasal cavity, with the cuff placed in the vestibulum of the nose with the head bent forward. After 10 min the fluid was recovered, and the samples filtrated through a 100 μm filter (Sintab, Malmö, Sweden), centrifuged (80 g for 10 min) and stored at −70°C. Cells were sampled from the nasal mucosa of the right nasal cavity by gentle nasal brushing (Irander *et al.* 1997).

Sample preparation

NLF samples were prepared for 2-DE as described previously (Lindahl *et al.* 1998). Cells were pelleted by centrifugation and the protein concentrations of the supernatants were determined by the Coomassie-based protein assay (Bio-Rad, Richmond, California, USA) according to the method of Bradford (1976). The protein concentrations were 60–760 μg ml^{-1} (median 120 μg ml^{-1}) in the controls before exposure, 80–1130 μg ml^{-1} (median 160 μg ml^{-1}) in the controls after exposure, 30–290 μg ml^{-1} (median 90 μg ml^{-1}) in the epoxy workers before exposure, 40–170 μg ml^{-1} (median 70 μg ml^{-1}) in the epoxy workers after exposure, and 60–330 μg ml^{-1} (median 130 μg ml^{-1}) in the smokers. Samples of 2.5 ml of the supernatant were desalted, lyophilized and dissolved in 0.25 ml urea solution suitable for the first dimension, according to Görg *et al.* (2000). In order to examine the number of eosinophils, material from the nasal brushing was shaken into a test tube containing 1 ml of phosphate buffered saline, pH 7.3. Aliquots of 0.2 ml from the test tube were cytocentrifuged (Cytospin 2, Shandon Inc., Pittsburgh, Pennsylvania, USA) onto a glass slide, air-dried and fixed in methanol for 10 min, and stained with Wright's stain. The specimens were coded to ensure a blind analysis, and the total number of cells or the number of eosinophils on the glass slide was recorded using light microscopy (magnification × 250) (Irander *et al.* 1997).

2-DE analysis

2-DE was performed in a horizontal 2-DE setup (Multiphor, Pharmacia, Uppsala, Sweden) as described previously (Lindahl *et al.* 1995, Görg *et al.* 2000). In the first dimension (isoelectric focusing), 15 μg protein per sample was applied in sample cups on immobilized pH gradient (IPG) strips (0.5 × 3 × 180 mm) containing Immobilines giving a non-linear pH gradient from 3 to 10 (Pharmacia, Uppsala, Sweden). To assure a steady state, the focusing was performed overnight (45 000 Vh). The second dimension – sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) – was carried out by transferring the proteins to ExcelGel XL SDS 12–14 from Pharmacia (0.5 × 180 × 245 mm) or to gradient gels cast on GelBond PAGE film (0.5 × 180 × 245 mm, 11–18% T, 1.5% C, 33–0% glycerol) running at 20–40 mA for about 4 h. In both cases ExcelGel SDS buffer strips from Pharmacia was used. Separated proteins were detected by silver staining with a detection limit of 1–5 ng/spot (Swain and Ross 1995). The isoelectric point and molecular mass of the separated proteins were estimated using pI and M_r standards (two-dimensional standards 4.5–8.5 and broad range 200–6.5 kDa, Bio-Rad) and reference values of known proteins in the samples (Bjellqvist *et al.* 1994).

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2-DE imaging process

The protein patterns in the gels, containing about 1000 spots, were evaluated as digitized images using a charge-coupled device (CCD) camera (1024 × 1024 pixels) in combination with a computerized imaging system designed for evaluations of 2-DE patterns (Visage 4.6, BioImage, Ann Arbor, Missouri, USA) (Lindahl *et al.* 1996). The amount of protein in a spot was assessed as background-corrected optical density, integrated over all pixels in the spot, and expressed as the integrated optical density (IOD). In order to correct for differences in total silver stain intensity between different 2-DE images, the amount of each protein was expressed as the percentage of the individual spot IOD per total spot IOD of the sample (%IOD). 2-DE patterns from different samples were compared by gel matching, in which 15–20 protein spots that appeared in all samples were selected and the software then corrected for spatial differences between different images and made a geometrical transformation (Lindahl *et al.* 1995). The matched gels were then used to evaluate qualitative and quantitative differences in patterns between different samples, individuals or groups of individuals. The variation in the quantitative determinations of a protein was less than 5%. Gel matching was also performed to demonstrate previously identified NLF proteins included in a human NLF and BALF 2-DE database (Lindahl *et al.* 1998).

N-terminal amino acid sequencing

About 800 μg of NLF proteins was applied by in-gel rehydration to the IPG strips in the first dimension. Following the second dimension, the proteins were transferred to polyvinylidene fluoride membranes by electroblotting as described previously (Lindahl *et al.* 1999a). After Coomassie blue staining, excluding acetic acid in the destaining step, about 300 spots were detected and their position translated to silver-stained gels and to our database identification number. The membrane was then dried and stored at −20°C until the protein spots were excised and the N-terminal amino acid sequences determined. The spots were applied to a Procise cLC or a Procise HT sequencer (PE-Applied Biosystems, Foster City, California, USA) for Edman degradation, enabling sequencing at the picomole level. The N-terminal amino acid sequence obtained was submitted to a database search (NCBI) using BLAST.

Peptide mass fingerprinting and post-source decay analysis with MALDI-TOF MS

About 300 μg of NLF proteins was separated with 2-DE. The gel was stained with 0.1% Coomassie brilliant blue R-250 for 1 h in 50% methanol and 1% acetic acid, and destained overnight in 30% methanol and 1% acetic acid. About 200 spots were detected and their position translated to silver-stained gels and to our database identification number. The gels were stored in destain solution in a coldroom until further preparation. The general principles for peptide mass fingerprinting and MALDI-TOF MS have been outlined elsewhere (Wilkins and Gooley 1997). In short, enzymatic cleavage of proteins with trypsin generates a peptide profile specific for each protein. The peptides are analysed with mass spectrometry and the spectrum generated is used for searches in gene and protein databases, allowing identification of the protein. In our protocol, the tryptic digest was obtained by washing the gel twice with water and the gel spot was excised with a razor blade, put in an Eppendorf tube and washed twice with 200 μl acetonitrile in 50 mM ammonium bicarbonate for 30 min, and then covered with 100 μl acetonitrile for 5 min. The acetonitrile was removed and the gel piece dried in a speed-vac (Savant, Farmingdale, New York, USA) for about 15 min. About 30 μl of 10 μg ml^{-1} trypsin (Promega, Madison, Wisconsin, USA) in 25 mM ammonium bicarbonate was added and the sample was incubated overnight at 37°C. The supernatant was then used for analysis of the peptide mass spectra, in which 0.5 μl of the digest was mixed 1:1 with matrix (5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid) and allowed to dry on the MALDI sample plate. In addition, 5 μl of the sample was cleaned with a C18 ZipTip according to the manufacturer's instructions (Millipore, Bedford, Massachusetts, USA) and eluted in 1.5 μl of matrix directly onto the MALDI plate. The sample was analysed with MALDI-TOF (Voyager-DE STR, PE-Biosystems or Bruker Reflex, Bruker Daltonics) in reflectron mode. Delayed extraction by an accelerating voltage of 20 kV was used and the sample was calibrated internally with tryptic autolysis peaks (842 and 2211 Da). A database search was performed using MS-Fit. Typical mass error was below 10 p.p.m. To confirm the identification, one peptide was selected and analysed by post-source decay (PSD) in which the laser power was increased and fragment spectra collected at decreasing mirror ratio voltages. The mass information generated from the composite spectrum was then submitted to a database search engine, MS-Tag or Mascot, together with precursor mass information and immonium ion information.

Statistical analysis

Values are given as the mean ± SD. The significance of the differences between healthy subjects (controls) and workers with airway irritation was calculated using the Mann–Whitney *U*-test, a non-parametric test for unpaired observations. The significance of the differences between before and after

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exposure was calculated using the Wilcoxon's signed rank test, a non-parametric test for paired observations.

Results

NLF 2-DE protein patterns in epoxy workers and controls

A typical 2-DE pattern is shown in figure 1. About 1000 proteins were detected in each NLF sample. There are distinct similarities, but also distinct differences, between these spot patterns and that of human plasma (Sanchez *et al.* 1995) and BALF (Lindahl *et al.* 1998). Albumin, transferrin and immunoglobulins are abundant in NLF, as in other extracellular fluids. Of the protein spots detected, about 35% have so far been identified and included in our NLF protein database (Lindahl *et al.* 1998). The complexity of the protein pattern is increased by the fact that most proteins are expressed as more than one form. This mainly reflects post-translational modifications such as glycosylations, phosphorylations and truncations.

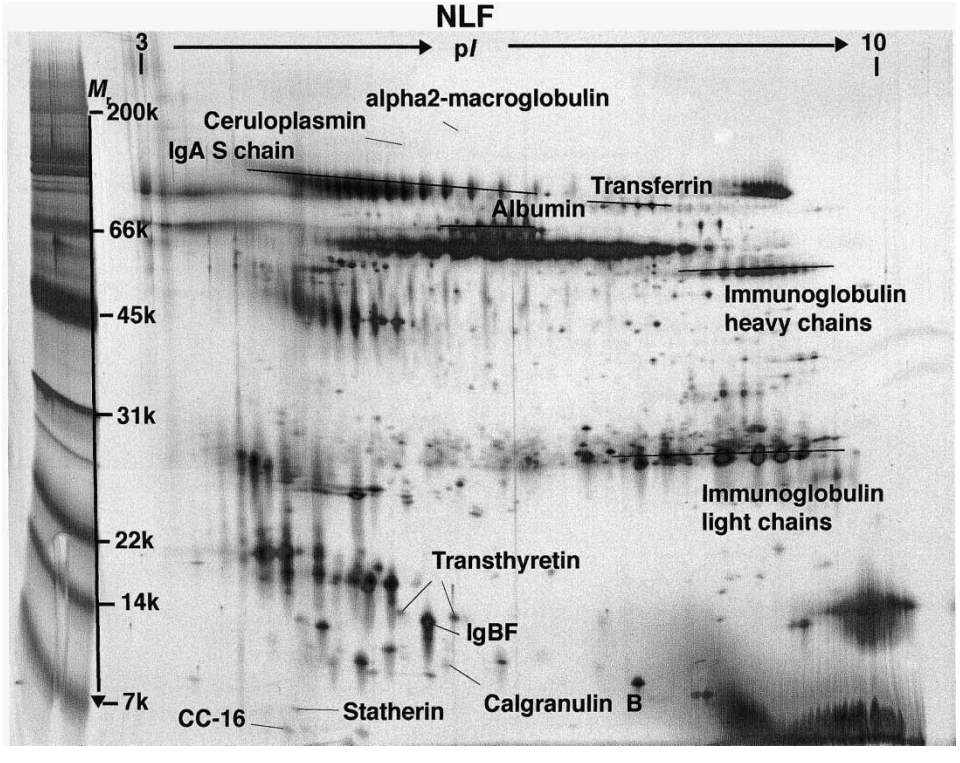

Figure 1. Typical two-dimensional gel electrophoresis (2-DE) protein pattern of nasal lavage fluid (NLF). 2-DE was performed in a non-linear Immobiline pH gradient from 3 to 10, followed by 12–14% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and separated proteins were detected by silver staining. Proteins whose levels changed after chamber exposure to dimethylbenzylamine, as well as some abundant NLF proteins, are indicated. The figure is a scanned gel presented as an unmanipulated image, except for overall contrast and light, using graphic processing programs (Photoshop 4.0 and PageMaker 6.5, Adobe Systems, San José, California, USA). IgA, immunoglobulin A; IgBF, immunoglobulin binding factor; CC-16, Clara cell protein 16.

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In NLF from the epoxy workers, lower levels of transthyretin, CC16 and α_2-macroglobulin than in controls were found (table 1). As a majority of the epoxy workers were smokers (six out of eight) and the controls comprised only non-smokers, NLF from seven smokers not exposed to DMBA were also investigated. It was found that the levels of CC16 and α_2-macroglobulin, but not of transthyretin, were lower in smokers than in non-smokers (table 1).

NLF 2-DE protein patterns after chamber exposure to DMBA

The positions of proteins whose levels were altered after chamber exposure are indicated in figure 1. In the controls, the levels of immunoglobulin A (IgA) increased and the levels of CC16 decreased, whereas in the epoxy workers, the levels of α_2-macroglobulin and caeruloplasmin increased (table 2).

One unknown protein (with an apparent M_r of 7 kDa and a pI of 4.8) whose level was decreased after chamber exposure in epoxy workers was identified as statherin (table 2). The N-terminal sequence determined (DSSEEKFLRRIG) was identical to a previously reported sequence in statherin (without signal sequence) in SWISS-PROT (accession number P02808).

A second unknown protein (with an apparent M_r of 9 kDa and a pI of 5.6) whose level was decreased after chamber exposure in epoxy workers was identified by MALDI-TOF MS as calgranulin B (table 1). Seven peaks in the trypsin spectrum (marked with an asterisk in figure 2) matched theoretical peptides in calgranulin B (SWISS-PROT accession number P06702) and the matched peptides covered 81% of the protein (table 3). Furthermore, according to the mass data, four peaks in the spectrum were most likely due to oxidized methionine residues in the protein. The mass deviation of the matched peptides between determined and expected ranged from 2 to 12 p.p.m. (table 3). One peptide with mass 1455.72 was selected and analysed by PSD. The expression sequence tag deduced (GHPDTLN) matched a sequence in the theoretical peptide with the same mass in calgranulin B (amino acid positions 27–33), thereby confirming the identity of the protein.

IgBF and eosinophil infiltration

Two healthy individuals and two epoxy workers developed high counts of eosinophils (>500 per nasal brush sample) in their nasal mucosa after 2 h' exposure to DMBA in the exposure chamber. As shown in table 4, this subgroup had significantly lower levels of IgBF before exposure than the 10 individuals with low infiltration of eosinophils (<100 per nasal brush sample). The levels of IgBF did not change significantly during exposure in either of the two groups. The identity of IgBF was confirmed by PSD analysis of a major peak from the trypsin spectrum (1798.8 Da). The expression sequence tag deduced (YFIP-NEGVPGDST) matched a sequence in the precursor peptide and the amino acid positions 23–35 in IgBF.

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Table 1. Protein levels in nasal lavage fluid (NLFs) from smokers ($n = 7$), epoxy workers (n = 8; two non-smokers and six smokers) and controls (n = 6) analysed using two-dimensional gel electrophoresis.

	Controls		Epoxy workers			Smokers			
Protein	%IOD	IOD	%IOD	IOD	Epoxy workers versus controls[a]	%IOD	IOD	Smokers versus controls[a]	Smokers versus epoxy workers[a]
Transthyretin	0.351±0.260	2.42±1.97	0.106±0.171	0.34±0.40	$p = 0.010$	0.234±0.216	1.10±0.49	$p > 0.250$	$p = 0.028$
Immunoglobulin A	1.465±1.060	10.99±9.25	1.922±1.743	9.64±11.37	$p > 0.250$	2.025±1.522	13.00±12.47	$p > 0.250$	$p > 0.250$
α_2-Macroglobulin	0.027±0.037	0.21±0.32	0.004±0.008	0.02±0.05	$p = 0.033$	0.008±0.010	0.04±0.05	$p = 0.200$	$p > 0.250$
Caeruloplasmin	0.016±0.029	0.15±0.24	0.035±0.045	0.16±0.17	$p > 0.250$	0.023±0.035	0.17±0.33	$p > 0.250$	$p > 0.250$
Clara cell protein 16	0.120±0.087	0.62±0.67	0.016±0.027	0.05±0.07	$p = 0.007$	0.021±0.020	0.13±0.14	$p = 0.010$	$p > 0.250$
Statherin	0.010±0.011	0.08±0.10	0.006±0.005	0.04±0.04	$p > 0.250$	0.016±0.010	0.08±0.03	$p = 0.123$	$p = 0.035$
Calgranulin B	0.023±0.018	0.15±0.10	0.014±0.014	0.05±0.05	$p = 0.245$	0.047±0.056	0.20±0.19	$p > 0.250$	$p = 0.180$

The nasal lavage fluid protein concentrations were 290±300 μg ml^{-1} in controls, 130±80 μg ml^{-1} in epoxy workers, and 150±100 μg ml^{-1} in smokers; 15 μg/sample were subjected to analysis. Values are the mean±SD.

IOD, integrated optical density; %IOD, percentage of the individual spot IOD per total spot IOD of the sample.

[a] Mann–Whitney *U*-test.

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Table 2. Protein levels in nasal lavage fluid from epoxy workers ($n = 8$) and controls ($n = 6$). Samples were taken before and after chamber exposure to dimethylbenzylamine and analysed using two-dimensional gel electrophoresis.

	Controls					Epoxy workers				
	Before exposure		After exposure			Before exposure		After exposure		
Protein	%IOD	IOD	%IOD	IOD	Controls after exposure versus before exposure[a]	%IOD	IOD	%IOD	IOD	Epoxy workers after exposure versus before exposure[a]
Transthyretin	0.351 ± 0.260	2.43 ± 1.97	0.262 ± 0.218	1.44 ± 1.10	$p > 0.250$	0.106 ± 0.171	0.34 ± 0.40	0.097 ± 0.074	0.37 ± 0.34	$p > 0.250$
Immunoglobulin A	1.465 ± 1.060	10.99 ± 9.25	2.290 ± 1.402	15.58 ± 10.64	$p = 0.046$	1.922 ± 1.743	9.64 ± 11.37	2.549 ± 1.946	12.18 ± 10.86	$p = 0.180$
α_2-Macroglobulin	0.027 ± 0.037	0.21 ± 0.32	0.029 ± 0.017	0.17 ± 0.10	$p > 0.250$	0.004 ± 0.008	0.02 ± 0.05	0.013 ± 0.017	0.06 ± 0.09	$p = 0.080$
Caeruloplasmin	0.016 ± 0.029	0.15 ± 0.24	0.015 ± 0.018	0.12 ± 0.14	$p > 0.250$	0.035 ± 0.045	0.16 ± 0.17	0.065 ± 0.064	0.35 ± 0.35	$p = 0.036$
Clara cell protein 16	0.120 ± 0.087	0.62 ± 0.67	0.037 ± 0.058	0.22 ± 0.35	$p = 0.028$	0.016 ± 0.027	0.05 ± 0.07	0.011 ± 0.017	0.04 ± 0.08	$p > 0.250$
Statherin	0.010 ± 0.011	0.08 ± 0.10	0.006 ± 0.010	0.04 ± 0.06	$p > 0.250$	0.006 ± 0.005	0.04 ± 0.04	0.003 ± 0.003	0.01 ± 0.02	$p = 0.018$
Calgranulin B	0.023 ± 0.018	0.15 ± 0.10	0.023 ± 0.026	0.14 ± 0.16	$p > 0.250$	0.014 ± 0.014	0.05 ± 0.05	0.005 ± 0.006	0.02 ± 0.02	$p = 0.063$

The nasal lavage fluid protein concentrations were 290 ± 300 μg ml^{-1} in controls before exposure, 330 ± 410 μg ml^{-1} in controls after exposure, 130 ± 80 μg ml^{-1} in epoxy workers before exposure, and 80 ± 50 μg ml^{-1} in epoxy workers after exposure; 15 μg/sample were subjected to analysis. Values are the mean ± SD.

IOD, integrated optical density; %IOD, percentage of the individual spot IOD per total spot IOD of the sample.

[a] Wilcoxon's signed rank test.

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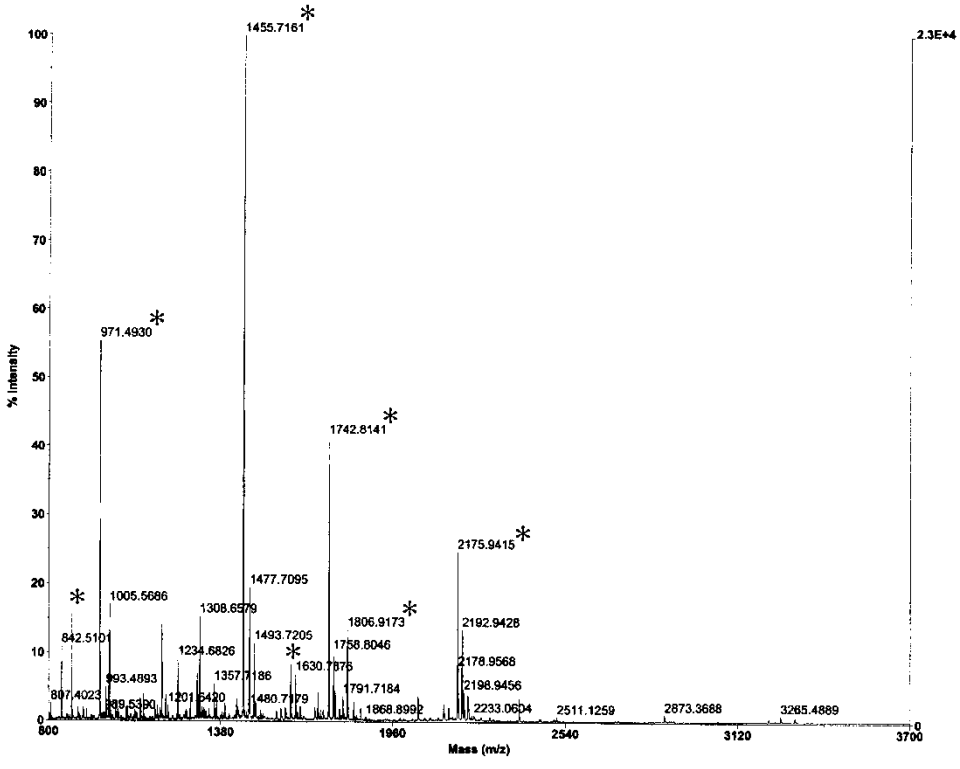

Figure 2. Peptide spectrum of a protein with an apparent M_r of 9 kDa and a pI of 5.6 whose levels decreased in nasal lavage fluid (NLF) from epoxy workers after chamber exposure to dimethylbenzylamine. NLF proteins were separated using two-dimensional gel electrophoresis and the protein analysed by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Peptides marked with an asterisk were matched to peptides in calgranulin B (table 2). m/z, mass-to-charge ratio.

Discussion

DMBA is a volatile and irritating chemical and some symptoms in the eyes and the upper respiratory tract would be expected after exposure. All the currently available data suggests that DMBA acts as a chemical irritant and there is no evidence for the involvement of any immune mechanisms. Little is known,

Table 3. Matched peptides in calgranulin B found after matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Determined mass (Da)	Expected mass (Da)	ΔDa	Start position	End position	Peptide sequence
877.476	877.478	−0.002	44	50	DLQNFLK
971.493	971.495	−0.002	86	93	LTWASHEK
1455.716	1455.723	−0.007	26	38	LGHPDTLNQGEFK
1614.788	1614.802	−0.014	73	85	QLSFEEFIMLMAR
1742.814	1742.827	−0.013	58	72	VIEHIMEDLDTNADK
1806.917	1806.939	−0.022	11	25	NIETIINTFHQYSVK
2175.942	2175.963	−0.021	94	114	MHEGDEGPGHHHKPGLGEGTP

Matched peptides covered 92 out of 114 amino acids (81%).

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Table 4. Levels of immunoglobulin binding factor in groups with low and high eosinophils before and after chamber exposure to dimethylbenzylamine.

	Low eosinophil group[a]		High eosinophil group[b]	
	Before exposure	After exposure	Before exposure	After exposure
Eosinophils/nasal brushing	20 ± 32	49 ± 48	25 ± 28	1060 ± 997**
Immunoglobulin binding factor				
%IOD	0.376 ± 0.194	0.409 ± 0.255	0.079 ± 0.039*	0.089 ± 0.073
IOD	2.07 ± 1.18	2.02 ± 1.36	0.46 ± 0.28	0.32 ± 0.19

Nasal lavage fluid and nasal brush samples were taken from 14 subjects (eight epoxy workers and six controls) and analysed using two-dimensional gel electrophoresis and differential cell counting, respectively. Values are the mean ± SD.

IOD, integrated optical density; %IOD, percentage of the individual spot IOD per total spot IOD of the sample.

[a] Low number of eosinophils per nasal brush sample (0–100); $n = 10$.

[b] High number of eosinophils per nasal brush sample (635–2550); $n = 4$.

* $p = 0.024$ for high eosinophil group before exposure versus low eosinophil group before exposure, Mann–Whitney U-test.

** $p = 0.005$ for high eosinophil group after exposure versus low eosinophil group after exposure, Mann–Whitney U-test.

however, about the effects caused by the chemical in the airways at a molecular level. By using a proteomic approach we were able to identify several proteins that were altered after short-term exposure to DMBA. This opens new possibilities to use NLF in order to get detailed information about protein changes that may be used as exposure/disease markers. The advantages of this type of approach have recently been reviewed by Kennedy (2002), and also used to examine BALF from patients with interstitial lung disorders (Noel-Georis *et al.* 2001). The procedure for collecting NLF is relatively simple and can also be used for field studies in occupational settings during the work-shift (Lindahl *et al.* 1995, Granstrand *et al.* 1998).

In this study, six of the eight epoxy workers, but none of the controls, were smokers. We have previously shown that smokers may have some alterations in their NLF protein patterns (Ghafouri *et al.* 2002). The differences in α_2-macroglobulin and CC16, but not transthyretin, shown in the present study between epoxy workers and controls before chamber exposure were probably due to the differences in smoking habits (table 1). It is therefore possible, and perhaps likely, that the response of the epoxy workers to DMBA in the exposure chamber could be influenced by their smoking habits. Our data show that the epoxy workers, when exposed to DMBA, showed upper airways symptoms and protein changes in the NLF, but it is not possible to differentiate whether these responses are influenced by their previous exposure to DMBA at work, by smoking habits, or by a combination of the two. However, the two epoxy workers who were non-smokers had the same response patterns as the smokers.

Protein levels were expressed as %IOD. If substantial alterations in the levels of abundant proteins (such as albumin) occurred, relative measurements may lead to the false impression of changes in the levels of other proteins. In the present

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investigation, however, expression of the data in absolute terms (IOD) showed essentially the same results as with %IOD (tables 1, 2 and 4).

In the epoxy workers the levels of caeruloplasmin and α_2-macroglobulin increased after chamber exposure. Both are high molecular weight proteins found in the circulation and it is possible that this reflects plasma exudation, one of the signs of inflammation (Vesterberg *et al*. 2001). Accordingly, this effect was seen only in the epoxy workers with airway irritation and not in the controls. It appeared to be an acute effect, since the levels of these two proteins were not higher in epoxy workers (and in the case of α_2-macroglobulin were in fact lower) than in controls before the chamber exposure. In the controls the levels of secretory IgA increased and the levels of CC16 (also known as CC10 and Clara cell secretory protein) decreased after exposure. Similar effects, but less pronounced, were also found in the epoxy workers. Notably, the levels of CC16 were already significantly lower in epoxy workers than in healthy controls before the chamber exposure, probably because of smoking habits. CC16 is an anti-inflammatory protein (Jorens *et al.* 1995) that is decreased in NLF and BALF from patients with asthma and rhinitis (Lindahl *et al.* 1999b, Lensmar *et al.* 2000) and in smokers compared with non-smokers (Ghafouri *et al.* 2002, Petrek *et al.* 2002). This protein has also been suggested as a sensitive marker of other forms of environmental toxic exposure (Bernard and Lauwerys 1995, Carbonnelle *et al.* 2002). Our results suggest that CC16, at least in NLF from non-smokers, could be used as a marker of airway irritation, and that decreased CC16 expression may play a role in the pathogenesis of asthma and rhinitis caused by occupational exposure to epoxy chemicals.

Among the NLF proteins altered in epoxy workers after chamber exposure to DMBA, two new proteins were identified: statherin and calgranulin B. Statherin is a salivary protein produced by submandibular and parotid glands. Our data showed that statherin is an abundant protein in NLF, indicating that the submucosal glands in the nose also produce statherin. The N-terminal sequence of statherin contains two serines in position two and three. According to the SWISS-PROT database (accession number P02808), these residues are likely to be phosphorylated. Our sequence analysis indicated that the serine in the third position was phosphorylated, as there was a drop in the amount of this residue. This is typically caused by the presence of phosphoserine that is eluted in the void volume of the liquid chromatography and therefore not detected in the specific serine peak. In saliva, phosphorylated statherin has been suggested to be important for the nucleation and growth of calcium minerals (Long *et al*. 2001). The function of statherin in the nose is unclear, but it has been reported as an anti-microbial protein in nasal secretions (Cole *et al.* 1999), and rheumatoid arthritis patients with sicca symptoms have low levels of salivary statherin (Jensen *et al.* 1997). It is possible that DMBA has a toxic effect on the nasal glands that could explain the decreased level of statherin in epoxy workers after exposure, although this is somewhat contradictory to the symptoms of hypersecretion often found in these subjects (Nielsen *et al.* 1994).

Calgranulin B, also known as myeloid-related protein-14 and S100 calcium-binding protein, is abundantly present in phagocytes but is also expressed constitutively in epithelial cells (van Heyningen and Dorin 1990, Kerkhoff *et al.* 1999). Increased levels of calgranulin B have been found in inflammatory disorders

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such as cystic fibrosis, rheumatoid arthritis and chronic bronchitis (Kerkhoff *et al.* 1998). The function of this protein is unclear, but it appears to be able to both stimulate and inhibit the activity of inflammatory cells (Donato 2001). In neutrophils, a complex of calgranulin B and calgranulin A is involved in the binding of fatty acids, especially arachidonic acid (Kerkhoff *et al.* 1999). With regard to the airways, calgranulin B has been found in sputum from patients with chronic obstructive pulmonary disease (Longbottom *et al.* 1992), and the anti-allergic drugs amlexanox and cromolyn bind to lung calgranulin B (Oyama *et al.* 1997). Our results show that calgranulin B is present in the upper airways as a secreted protein and that the levels are decreased after exposure to DMBA. It is possible that this response is mediated through a direct effect on resident macrophages or epithelial cells or indirectly through some of the other proteins that were affected by the exposure. Although DMBA is not considered particularly reactive towards proteins, it is also possible that decreased levels of calgranulin B, as judged by 2-DE, are caused by the formation of a DMBA–calgranulin B adduct.

We have previously found increased numbers of eosinophils in the nasal mucosa of healthy individuals exposed to DMBA for 2 h (Irander *et al.* 1997), and the same response has also been seen in epoxy workers (unpublished observations). Recently, we identified IgBF in NLF with N-terminal sequencing (Lindahl *et al.* 1999a), and the identity of this protein has now been confirmed with PSD analysis. Moreover, we found that four individuals with particularly high numbers of eosinphils after chamber exposure to DMBA had significantly lower levels of IgBF compared with the others, indicating that eosinophil infiltration may be under the control of IgBF. The function of IgBF is not known but, since it binds to immunoglobulins and suppresses B-cell activation, it has been suggested as a modulator of local immune responses (Kamada *et al.* 1998). IgBF has also been demonstrated in BALF, and immunohistochemical staining suggests that it is produced in mucous glands and goblet cells (Ogushi *et al.* 1995). As eosinophils are regarded as key cells in asthma and rhinitis, the finding that IgBF levels appear related to eosinophil infiltration is worthy of further investigation.

The specific roles(s), if any, of statherin, calgranulin B and IgBF in airway inflammation caused by DMBA exposure can only be speculative. We have recently reported on another abundant NLF protein, PLUNC, which binds to lipopoly-saccharide and is also influenced by DMBA exposure (Ghafouri *et al.* 2003). Interestingly, statherin, calgranulin B, IgBF and PLUNC are all tentative antibacterial proteins (Cole et al. 1999, 2002). The host defence system for the recognition of bacteria is believed to consist of two pathways, one that promotes direct killing of bacteria and another that promotes an inflammatory response. Thus, it is possible that exposure to irritants that downregulates antibacterial proteins causes a more pro-inflammatory state in the airways.

In conclusion, we have identified distinct protein changes in NLF after 2 h' exposure to low levels of the irritating epoxy chemical DMBA. In particular, three novel proteins – statherin, calgranulin B and IgBF – were found to be altered. These proteins have not been described before in relation to upper airway problems due to irritant exposure. The proteins affected may serve as irritation markers, and further investigations will show if they occur after exposure to irritants in general.

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The levels of the proteins identified in this study were about 0.01–3 μg ml^{-1} NLF, and changes of the order of two- to seven-fold were recorded. The clinical relevance of these changes is yet unclear and these data alone do not allow conclusions concerning inflammatory diseases such as asthma and rhinits. However, further studies on the functions of statherin, calgranulin B and IgBF, and their relationship to inflammatory reactions such as the formation of cytokines may give new insight into the mechanisms involved in airway inflammatory processes.

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