

Modifications of *Phleum pratense* Grass Pollen Allergens following Artificial Exposure to Gaseous Air Pollutants (O₃, NO₂, SO₂)

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Key Words

Traffic-related pollution • Grass pollen allergens • Two-dimensional electrophoresis • Ozone • Nitrogen dioxide • Sulphur dioxide

Abstract

Background: Air pollution is frequently proposed as a potential cause of the increased incidence of allergy in industrialised countries. Our objective was to investigate the impact of the major gaseous air pollutants on grass pollen allergens. **Methods:** Timothy grass pollen was exposed to ozone (O₃), nitrogen dioxide (NO₂) and sulphur dioxide (SO₂) alone or in combination. Allergen contents were analysed by 2-dimensional immunoblot using grass pollen-sensitive patient sera. **Results:** For O₃-treated pollen, immunoblotting showed an acidification of allergens Phl p 1b, Phl p 4, Phl p 5 and Phl p 6 and an IgE recognition decrease in Phl p 1, Phl p 2, Phl p 6 and Phl p 13. NO₂ exposure induced a decrease in Phl p 2, Phl p 5b and Phl p 6 recognition, and SO₂ treatment induced a decrease in Phl p 2, Phl p 6 and Phl p 13 recognition. Moreover, samples treated with a mix of NO₂/O₃ or NO₂/SO₂ showed a higher decrease in allergen content, compared with samples treated with

only one pollutant. The O₃ acidification was also observed with the NO₂/O₃ mix. **Conclusion:** Exposure of pollen to gaseous pollutants induced a decrease in allergen detection in pollen extracts. This decrease could be due to a mechanical loss of allergens from the altered pollen grains and/or post-translational modifications affecting allergen recognition by IgE.

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Introduction

Respiratory allergic diseases are increasing worldwide in both prevalence and severity, affecting in particular subjects living in urban areas [1, 2]. Although the role played by air pollution has yet to be clarified, a body of evidence suggests that urbanisation, with its high levels of transport emissions, and a westernised lifestyle are linked to the rising frequency of these diseases observed in most of the industrialised countries [3–5]. Atmospheric pollution is a complex mixture of gaseous and particulate compounds interacting with each other. The main classical gaseous air pollutants are nitrogen dioxide (NO₂), ozone (O₃) and sulphur dioxide (SO₂). Table 1 summarises the main sources and European norms for atmospheric concentrations for each of these pollutants, compared with the doses used in our study.

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Table 1. Sources and comparison of European norms and doses used of gaseous pollutants (NO₂, O₃, SO₂)

Pollutant	Source (main season concerned)	European norms	Doses used in the study
NO ₂	All the year oxidation of NO emitted during high-temperature combustion of fuels (power station and transport roads)	52 µg/m ³ (average over 1 year) warning level: 400 µg/m ³ (3 h)	2 ppm (4 h): 3,800 µg/m ³
O ₃	Summer secondary reaction between volatile organic compounds and NO _x under UV	120 µg/m ³ (average over 8 h) information level: 180 µg/m ³ warning level: 240 µg/m ³	0.1 ppm (4 h): 200 µg/m ³
SO ₂	Winter combustion of fossil fuels containing sulphur (mainly from power stations)	20 µg/m ³ (average over 1 year) warning level: 500 µg/m ³ (3 h)	2 ppm (4 h): 5,300 µg/m ³

Laboratory studies confirmed the epidemiological evidences that inhalation of some pollutants, either individually or in combination, adversely affects lung function in asthmatics [6–9] and increases asthma severity [10–12]. However, the exact nature and role of outdoor pollutants in allergic sensitisation is still not entirely clear [13].

Alternatively, air pollution can directly affect pollen grains. For example, exposure to pollutants (CO, NO₂ and SO₂ at 10,000 ppm each) of 3 tree species and 1 grass pollen has been shown to alter the allergen content [14, 15]. After exposure of *Phleum pratense* pollen to high concentrations of SO₂ (16.5 mg/m³ = 7 ppm), but not to NO₂ (1 mg/m³ = 1 ppm), Phl p 5 recognition using monoclonal antibody (mAb) Bo1 was significantly reduced depending on exposure duration [16]. Pollen that had been collected at road sides showed significantly reduced allergen release in comparison with pollen collected in rural meadows [17]. Moreover, morphological modifications were reported in pollen shape and tectum under polluted conditions [18, 19]. Pollen grains collected from polluted areas were covered with large amounts of pollutants such as airborne particles, heavy metals, nitrate and sulphur [16]. Furthermore, our laboratory experiments have shown that NO₂ (0.5–50 ppm) and O₃ (0.1–5 ppm) treatments can alter pollen tectum and facilitate intracytoplasmic granule release in a dose-dependent manner [20]. These granules, which contain most of the pollen major allergens, have been implicated in thunderstorm-associated asthma [21, 22]. They have also been shown to trigger primary and secondary allergic responses in pollen-sensitised Brown Norway rats [23, 24]. Recently, *Zinnia* pollen exposed to polluted air has been shown to trigger higher allergic responses in guinea pig [18].

The aim of the present study was to characterise modifications of *P. pratense* pollen allergenic content following artificial exposure to the major gaseous air pollutants O₃ (0.1 ppm), NO₂ (2 ppm) and SO₂ (2 ppm). Based on our previous study [20], the pollutant concentrations used were the ones inducing the lowest increase in damaged pollen and intra-cytoplasmic granule release compared with air-exposed pollen. Compared with the ones used in most of the studies, these low concentrations were up to 10 times the European warning levels (table 1). Differing from other previous similar studies, an original fluidised bed exposure system was used in our study in order to mimic environmental exposures of pollen, and allergens were detected using sensitive patient sera after separation by 2-dimensional (2D) PAGE electrophoresis.

Material and Methods

Pollen and Pollen Extract

Pollen from *P. pratense* (timothy grass) and *Dactylis glomerata* (orchard grass) were purchased from Allergon AB (Angelholm, Sweden) and stored at 4°C (non-exposed pollen). For pollen extracts, 50 mg of pollen was suspended in 500 µl of distilled water, incubated on a rotating drum for 1 h at room temperature and then centrifuged for 10 min at 10,000 g [25]. The supernatant was stored at –20°C until use. Protein concentrations were assessed as described by Bradford [26] and Lowry et al. [27] using bovine serum albumin as a standard.

Pollen Exposure to Pollutants in Fluidised Bed

The exposure system consisted of a glass cylinder (250 mm in height, 20 mm in diameter) with a porous glass plate at the bottom. Two grams of *P. pratense* pollen was placed in the cylinder and formed a suspension of fine particles 25–30 mm thick from the porous glass plate. Dry air alone or with pollutants (0.1 ppm

O₃, 2 ppm NO₂, 2 ppm SO₂, the mix of 0.1 ppm O₃ and 2 ppm NO₂, or the mix of 2 ppm NO₂ and 2 ppm SO₂) was injected from the bottom at a constant rate of 1.2 l/min for 4 h. O₃ was generated by passing oxygen through an UV ioniser (Sonimix 6023, LN Industries SA, Geneva, Switzerland). Ozone concentration was monitored inside the exposure system by an O₃ analyser (O₃ 41M, Environnement SA, France) and adjusted by modifying the UV lamp intensity. Concentrations of NO₂ and SO₂ were obtained from gas containers prepared and certified by the supplier (Air Liquide, France). Homogenous pollen exposure was obtained by a slight contact of the cylinder with a vortex [20].

2D PAGE

Proteins from pollen extracts were separated by isoelectric focusing in a 4% polyacrylamide gel (CleanGel IEF, GE Healthcare AB, Uppsala, Sweden) containing 2% (w/v) Servalyt®, pH 2–11 (Serva, Heidelberg, Germany), in a Multiphor II electrophoresis chamber (GE Healthcare AB). Two adjacent 4-mm-wide strips of a focused protein extract were cut and submitted side by side to an SDS-PAGE separation on an 8–18% gradient gel (ExcelGel, GE Healthcare AB), given 2 quasi identical 2D separations. One of the 2D gel separation was silver stained according to Blum et al. [28] and the other used for an immunoblot.

Immunoblots

After 2D gel electrophoresis, proteins were electroblotted onto a nitrocellulose (NC) sheet (Schleicher & Schull, Basel, Switzerland) with a semi-dry Novablot apparatus, according to the manufacturer's instructions (1 h 15 min, 1 mA/cm²). All following incubations were performed using phosphate-buffered saline (PBS) containing 0.3% Tween-20 (PBS-Tw) at room temperature (about 22°C). NC sheets were blocked for 1 h with PBS-Tw and incubated (overnight) with either grass pollen-allergic patient sera (1:10) or mouse mAb directed against *D. glomerata* allergen 4 (Dac g 4), revealing also Phl p 4 (1:1 500) [29], or rabbit polyclonal antibody directed against rDac g 3 (1:500) [30]. Phl p 3 can be easily identified by the polyclonal antibody directed against rDac g 3 due to their sequence homology in grass pollen family [31]. After extensive washing, the NC sheets were incubated for 2 h with either alkaline phosphatase (AP)-conjugated goat anti-human IgE (1:700; Sigma-Aldrich, St. Louis, Mo., USA), AP-conjugated rabbit anti-mouse IgG (1:10 000; Sigma-Aldrich) or AP-conjugated mouse anti-rabbit IgG (1:5,000; Sigma-Aldrich). AP activity was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma-Aldrich) in 0.1 mol · l⁻¹ Tris buffer, pH 9.5.

Patient Sera

Sera No. 10, 12 and 25 were selected from a group of 26 grass pollen-allergic donors for their ability to recognise a great number of grass pollen allergens after blotting [32].

Results

Non-Exposed and Air-Exposed Pollen

To study the effect of gaseous pollutants on proteins and allergens, 2D separations were performed on water-soluble

extract from non-exposed and exposed *P. pratense* pollen. The 2D gel silver staining of non-exposed protein extract revealed about 30–40 proteins with a large spectrum of relative molecular masses (Mr) and isoelectric points (pI) (fig. 1a). Most of the proteins had an Mr comprised between 14 and 77 kDa and a pI between 3.5 and 10.6.

The 2D immunoblot patterns of non-exposed timothy grass pollen extract obtained using the 3 patient sera were similar. Patient serum No. 10 revealed about 30 allergen spots, listed in the www.allergome.com database, with an Mr range from 14 to 90 kDa and a pI range from 3.5 to 10.6 (fig. 1b). Allergens were mainly located around 5 different Mr ranges: 75–90, 65–70, 40–57, 25–26 and 17–24 kDa, each showing a great pI variability. Three spots at 75–90 kDa, pI 3.2–3.9 (unknown high molecular mass allergen), named [a], and 5 spots at 65–70 kDa, pI 6.4–8.3, were revealed and could correspond to Phl p 13 isoforms. Using a mAb against Dac g 4 which also reveals Phl p 4, 1 spot (around 70 kDa, pI 9.5–10.0) was recognised (data not shown). Two acidic allergen spots (pI 5.0–5.7) and 3 other acidic spots (pI 4.8–5.7), 6 neutral (pI 5.8–8.0) and 1 basic one (pI 10.0) were strongly revealed with Mr ranges of 40–57 kDa. They could correspond to Phl p 1, Phl p 5a, Phl p 5b and Phl p 1b, respectively. Acidic allergen spots (from pI 4.0 to 5.9) were also detected at Mr 25–26 and 17–24 kDa, probably corresponding to Phl p 6 and Phl p 2, respectively. Moreover, at Mr 17–24 kDa, basic spots (from pI 7.0 to 9.0), named Phl p 3, visualised by the patient serum No. 10, were also recognised by the anti-Dac g 3 antibodies. This serum weakly recognised a very acidic allergen (pI around 3.0) with low Mr (14–20 kDa) corresponding to profilin (Phl p 12). Compared with other tested sera, this one recognised the greatest allergen number and most of the known allergens.

Serum No. 25 did not reveal Phl p 1b and Phl p 4 but recognised all other allergens revealed by serum No. 10 (data not shown). Serum No. 12 mainly recognised major allergens, e.g., Phl p 1 and Phl p 5 (fig. 1d). Allergen patterns of aqueous extract from non-exposed timothy grass pollen, revealed by IgE from patient sera No. 10 and 25, were similar to those of orchard grass pollen extract (data not shown).

2D blots performed with extract from air-exposed pollen (fig. 1c) did not show any significant difference compared with non-exposed pollen extract (fig. 1b).

Gas-Exposed Pollen

Proteins from Different Pollen Extracts

Timothy grass pollen grains were exposed to air, O₃, NO₂ or SO₂, alone or in combination. Using the methods

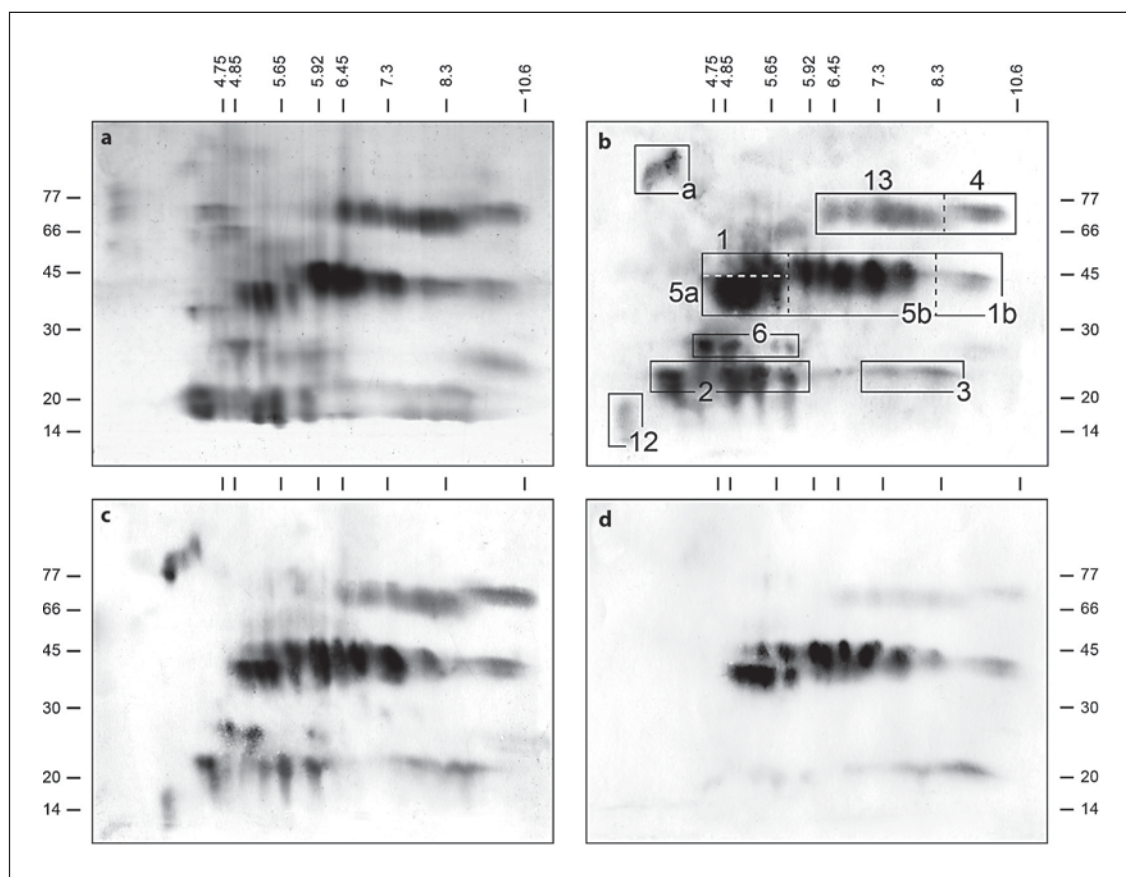


Fig. 1. 2D gel analysis of water-soluble extract from non-exposed and air-exposed *P. pratense* pollen. **a** Silver staining of proteins from non-exposed pollen. 2D immunoblots from non-exposed (**b**) and air-exposed (**c**) pollen revealed by IgE from grass pollen-sensitive patient serum No. 10 and non-exposed pollen (**d**) revealed by serum No. 12. Each *P. pratense* allergen (Phl p) is named (Allergome database) and shown in a squared area in the non-exposed blot (**b**). pI (at the top) and Mr (on the left and right) are indicated for each gel.

by Bradford [26] and Lowry et al. [27], protein amounts in water-soluble pollen extracts showed no significant difference between air-exposed and gaseous pollutant-exposed pollen. By the Bradford method, protein concentrations were 51.1 ± 3.7 , 51.9 ± 3.6 , 48.8 ± 8.0 , 51.9 ± 4.0 μg of protein/mg of pollen for air-, O_3 -, NO_2 - and SO_2 -exposed pollen extract, respectively.

2D Protein Patterns

No significant differences in the 2D protein patterns were observed between non-exposed (fig. 1a), air-exposed and NO_2 and/or SO_2 -exposed pollen extracts (data not shown), while O_3 treatment induced acidification of some proteins without visible change in silver staining intensity (fig. 2a).

2D Allergen Pattern of O_3 -Exposed Pollen

Acidic shift was also observed at the allergen level with the 3 sera. For example, with serum No. 10 (fig. 2b), allergen Phl p 4 and Phl p 1b switched from pI 10.0 to 8.3. Similarly, the 2 remaining acidic spots corresponding to Phl p 5a and Phl p 6 switched from pI 4.8–5.7 to 4.4–4.8 and from pI 4.0–5.9 to 4.3–4.8, respectively. In addition to this allergen acidification, a decrease in IgE recognition was observed with all sera. At Mr 75–90 kDa, the patient serum No. 10 (fig. 2b) revealed only an acidic spot around pI 3.2 corresponding to allergen [a]. Some of the Phl p 13 spots in the pI 7–8 area have disappeared. Concerning Phl p 1 and Phl p 5a, IgE recognition decreased and the Phl p 1b strongly diminished compared with non-exposed pollen extract. Moreover, Phl p 6 isoforms with pI 5.7–5.8 disappeared, and in the

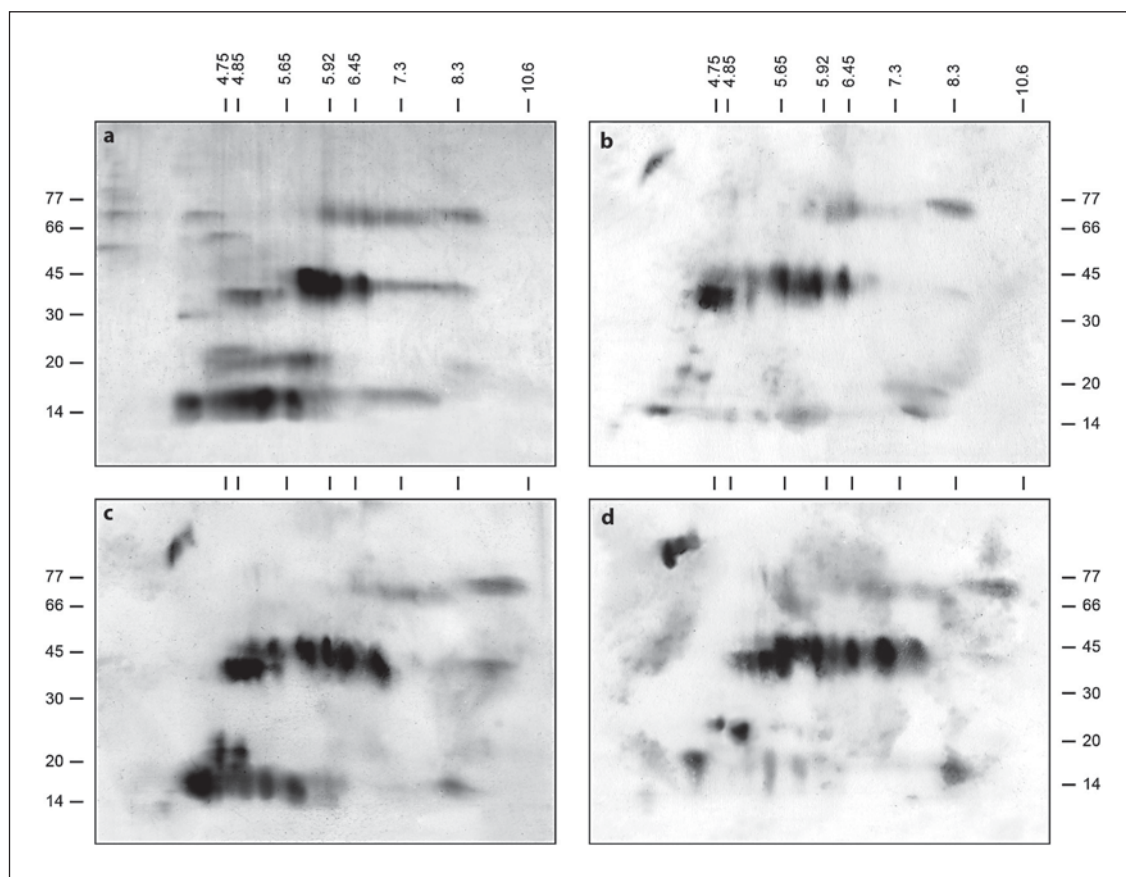


Fig. 2. 2D gel analysis of water-soluble extracts from O_3 -, NO_2 - and SO_2 -exposed *P. pratense* pollen. Pollen grains were exposed during 4 h to 0.1 ppm O_3 , 2 ppm NO_2 or 2 ppm SO_2 . **a** Silver staining of proteins from O_3 -exposed pollen. 2D immunoblots of O_3 - (**b**), NO_2 - (**c**) and SO_2 -exposed pollen allergens (**d**) revealed by IgE from grass pollen-sensitive patient serum No. 10. pI (at the top) and Mr (on the left and right) are indicated for each gel.

Phl p 2 area (Mr 17–24 kDa), a large decrease in isoform numbers was shown.

2D Allergen Pattern of NO_2 -Exposed Pollen

Using the 3 sera, 1 of the Phl p 5b spots (pI 7.3–7.5) was not detected in the pattern from NO_2 -exposed pollen (fig 2c; data not shown). Using sera No. 10 and 25, 2 of the Phl p 6 spots (pI 5.7–5.8) have disappeared and 1 of the Phl p 2 spots (pI 5.7–5.8) was more weakly revealed than in the control extract. No other differences were observed.

2D Allergen Pattern of SO_2 -Exposed Pollen

SO_2 treatment induced a decrease in IgE recognition of several allergens using the 3 sera. With serum No. 10 (fig. 2d), Phl p 13 (pI 6.4–8.3), Phl p 6 (pI 5.7–5.8) and Phl

p 2 (pI 4.0–5.9) recognitions were strongly decreased when recognitions of major allergens Phl p 1 and Phl p 5 showed no significant difference. However, using serum No. 12, we observed a decrease in Phl p 1 and Phl p 5 recognition (data not shown).

2D Allergen Pattern of Gas Mixture Exposed-Pollen

The allergen patterns obtained with serum No. 10 from combined gaseous pollutant-exposed pollen extracts are shown in figure 3. A strong reduction in IgE recognition was observed for the whole pattern from NO_2/O_3 -exposed pollen extract (fig. 3a). In spite of similar allergen patterns, the reduction was slightly more pronounced on NO_2/O_3 -exposed pollen (fig. 3a) than on O_3 -exposed pollen (fig. 2b). Allergen acidification shown with O_3 treatment was also observed in this case. The re-

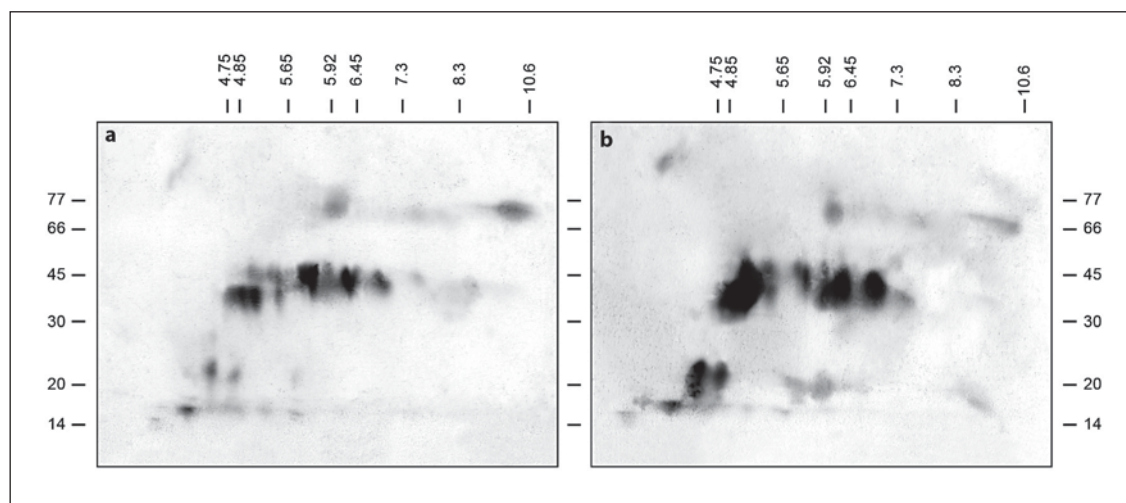


Fig. 3. 2D gel analysis of water-soluble extracts from NO₂/O₃- and NO₂/SO₂-exposed *P. pratense* pollen. Pollen grains were exposed during 4 h to a mixture of either 2 ppm NO₂ and 0.1 ppm O₃ (**a**) or 2 ppm NO₂ and 2 ppm SO₂ (**b**). 2D immunoblots of NO₂/O₃- (**a**) and NO₂/SO₂-exposed pollen allergens (**b**) revealed by IgE from grass pollen-sensitive patient serum No. 10. pI (at the top) and Mr (on the left and right) are indicated for each gel.

sults previously described were obtained with sera No. 12 and 25 as well (data not shown).

In comparison with single pollutant-exposed pollen (fig. 2c, d), allergen pattern from NO₂/SO₂-exposed pollen (fig. 3b) revealed a significant decrease in high Mr allergens: [a], Phl p 4 and Phl p 13. The recognition of the other allergens was similar to the one obtained after SO₂ treatment (fig. 2b).

Discussion

When timothy grass pollen exposures were performed with realistic O₃ concentration and/or with NO₂ and SO₂ concentrations 10 times higher than the European warning level, we observed a decrease in some allergen recognition by IgE from 3 allergic patient sera. Acidification of several allergens was also observed following treatment using O₃ with or without NO₂. These experimental gaseous exposures did not induce any apparition of de novo allergens. The decrease in allergen recognition suggests either allergen loss during the treatment (for example the release into the atmosphere as free allergens) and/or their alteration.

Protein and allergen losses due to urban pollution exposure or to gaseous experimental exposure to high concentrations of pollutants were previously described [18, 19, 25]. However, in our experiments, we used lower pol-

lutant concentrations than those used by the former authors, inducing few damages on pollen grains and a weak intra-cytoplasmic granule release [20]. In these conditions, we have not observed any noticeable difference between air- and gaseous pollutant-exposed pollen on protein concentration and on silver-stained 2D gel profiles. The 'allergen loss' hypothesis should be less suitable on the basis of our results but further work is needed. For example, pollen cytoplasmic granules and free allergen release could be measured following pollen exposures to gaseous pollutants.

Air pollutants could have an effect on the biochemical protein properties of pollen grains which might induce a decrease in the allergen recognition by IgEs. Goschnick et al. [33] analysed the effect of NO₂ (1–100 ppm) or O₃ (0.1 ppm) treatment on 3 species of pollen (timothy grass, mugwort and birch), showing incorporation of nitrogen as nitro groups into the organic matrix of the pollen wall. Recently, Franze et al. [34] reported pollen protein nitrations, in particular on birch allergen Bet v 1, after exposure to a mixture of NO₂ (4.57 ppm) and O₃ (0.5 ppm). After exposure to this gas mixture, the level of protein nitration depends on the concentration of O₃ and NO₂ and their highly reactive species (NO₃ or HNO₃) [34]. Furthermore, post-translational modifications, such as hydroxylated proline residues, carbohydrate structures and disulfide formation [35] or deglycosylation [36], can affect the IgE reactivity. Also, an alteration of the major

phosphorylation site of an important allergenic epitope of casein affects the allergenicity of this protein [37]. In our experiments, we hypothesise that the observed decrease in IgE recognition induced by gaseous pollutant exposures could be due to a post-translational modification as previously described [33–35]. Identification of the allergen composition by mass spectrometry should allow the characterisation of chemical modifications that may be responsible for the decrease in allergen recognition. It could be interesting to investigate the role of these chemical modifications in the modified pollen allergenicity in vivo. For this purpose, we have studied the allergenicity of these polluted grass pollens in sensitised Brown Norway rats and, until now, we failed to observe any significant modification of the specific humoral (pollen IgE) and cellular (lymphocyte proliferation in response to pollen) allergic responses (unpublished data).

In conclusion, the pollen exposure to gas pollutants induces a slight decrease in allergen detection in pollen extracts. This decrease could be due to post-translational modifications affecting allergen detection by IgE patient sera. Our preliminary results on Brown Norway rats emphasise the difficulty to evaluate the impact of these allergen modifications on the allergenicity in vivo. Knowledge of this phenomenon would require further studies with different animal models of allergy.

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