Modifications of Phleum pratense grass pollen allergens following artificial exposure to gaseous air pollutants (O3, NO2, SO2)

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Abstract

Background: Air pollution is frequently proposed as a potential cause for 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 O_3, NO_2, SO_2 alone or mixed. Allergen contents were analysed by 2D-immunoblot using grass pollen-sensitive patient sera.

Results: For O_3-treated pollen, immunoblotting showed an acidification of allergens Phl p 1b, 4, 5 and 6 and an IgE recognition decrease in Phl p 1, 2, 6, 13. NO_2-exposure induced a decrease in Phl p 2, 5b and 6 recognition and SO2-treatment induced a decrease in Phl p 2, 6 and 13 recognition. Moreover, samples treated with a mix of NO_2 / O_3 or NO_2 / SO_2 showed a higher decrease in allergen content, compared to samples treated with only one pollutant. The O_3 acidification was also observed with the mix NO_2/O_3.

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.

Introduction

Respiratory allergic diseases are increasing world-wide 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 (NO_2), ozone (O_3) and sulphur dioxide (SO_2). Table I summarise for each one, the main sources and European norms for atmospheric concentrations compared to the doses used in our study.

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. As an example, exposure to pollutants (CO, NO_2 and SO_2 at 10,000 ppm each) of 3 tree species and 1 grass pollens, has been shown to alter the allergen content[14, 15]. After exposure of *P. pratense* pollen to high concentrations of SO_2 (16.5 mg/m^3=7 ppm), but not to NO_2 (1 mg/m^3=1 ppm), Phl p 5 recognition using 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, sulphur[16]. Furthermore, our laboratory experiments have shown that NO_2 (0.5 to 50 ppm) and O_3 (0.1 to 5 ppm) treatments can alter pollen tectum and facilitate intra-cytoplasmic granules release, in 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, *Zinia* 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 O_3 (0.1 ppm), NO_2 (2 ppm) and SO_2 (2 ppm). Based on our previous study[20], the pollutant concentrations used were the ones inducing the lowest increase of damaged pollen and intra-cytoplasmic granules release compared to air-exposed pollen. Compared to the ones used in most of the studies, these low concentrations were up to 10 times the European warning levels (Table I). 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 2D-PAGE electrophoresis.

Material and methods

Pollen and pollen extract

Pollen from *Phleum 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 were suspended in 500 microL of distilled water, incubated on a rotating drum for 1 hour at room temperature and then centrifuged for 10 min at 10,000 g[25]. The supernatant was stored at –20°C until

use. Proteins concentrations were assessed as described by Bradford[26] and by Lowry *et al.* [27] using bovine serum albumin as a standard.

Pollen exposure to pollutants in fluidized-bed

The exposure system consisted of a glass cylinder (250-mm height, 20-mm diameter) with a porous glass plate at the bottom. Two grams of *P. pratense* pollen were placed in the cylinder and formed a suspension of fine particles 25 to 30 mm thick from the porous glass plate. Dry air alone or with pollutants (0.1 ppm O_3, 2 ppm NO_2, 2 ppm SO_2, the mix of 0.1 ppm O_3 and 2 ppm NO_2 or the mix of 2 ppm NO_2 and 2 ppm SO_2) were injected from the bottom at a constant rate of 1.2 L/min, for 4 hours. O_3 was generated by passing oxygen through an UV-ionizer (Sonimix 6023, LN Industries SA, Geneva). Ozone concentration was monitored inside the exposure system by an O_3 analyzer (O_3 41M, Environnement SA, France) and adjusted by modifying the UV lamp intensity. Concentrations of NO_2 and SO_2 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 isoelectricfocusing (IEF) 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 (AB). Two adjacent 4-mm-wide strips of a focused protein extract were cut and submitted side by side to a SDS-PAGE separation on a 8-18% gradient gel (ExcelGel, AB), given 2 quasi identical 2D separation. One of the 2-D gel separation was silver-stained according to Blum *et al.*[28] and the other used for an immunoblot.

Immunoblots

After 2-D 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 (1h15, 1 mA/cm^2). 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 1 hour with PBS-Tw and incubated (overnight) with either grass pollen-allergic patient sera (1:10), or mouse monoclonal antibody (mAb) directed against *Dactylis glomerata* allergen 4 (Dac g 4), revealing also Phl p 4[29] (1:1 500) or rabbit polyclonal antibody directed against rDac g 3[30] (1:500). 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 2 hours with either alkaline phosphatase (AP)-conjugated goat anti-human IgE (1:700, Sigma-Aldrich, St. Louis, USA), or 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 mole.L^{-1} Tris buffer, pH 9.5.

Patient sera

Sera number 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:

1. Non-exposed and air-exposed pollen

To study the effect of gaseous pollutants on proteins and allergens, two-dimensional separations were performed on water-soluble extract from non-exposed and exposed *P. pratense* pollens. 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 a Mr comprised between 14 and 77 kD and a pI between 3.5 and 10.6.

The 2D immunoblot patterns of non-exposed timothy grass pollen extract obtained using the three patient sera were similar. Patient serum #10 revealed about 30 allergen spots, named in www.allergome.com database, with a Mr range from 14 to 90 kD and a pI range from 3.5 to 10.6 (fig 1b). Allergens were located mainly around five different Mr ranges: 75-90, 65-70, 40-57, 25-26 and 17-24 kD, each of them showing a great pI variability. Three spots at 75-90 kD, pI 3.2-3.9 (unknown high molecular mass allergen) named [a] and 5 spots at 65-70 kD, pI 6.4-8.3 were revealed and could correspond to Phl p 13 isoforms. Using a mAb against Dac g 4 which reveals also Phl p 4, one spot (around 70 kD, pI 9.5-10.0) was recognised (data not shown). Two acidic allergen spots (pI 5.0-5.7) and 3 other acidic (4.8-5.7), 6 neutral ones (pI 5.8-8.0) and 1 basic one (pI 10.0) were strongly revealed with Mr ranges of 40-57 kD. They could correspond to Phl p 1, 5a, 5b and 1b respectively. Acidic allergen spots (from pI 4.0 to 5.9) were also detected at Mr 25-26 and 17-24 kD, probably corresponding to Phl p 6 and Phl p 2 respectively. Moreover, at Mr 17-24 kD, basic spots (from pI 7.0 to 9.0), named Phl p 3, visualised by the patient serum #10, were also recognised by the anti-Dac g 3 antibodies. This serum recognised weakly a very acidic allergen (pI around 3.0) with low Mr (14-20 kD) corresponding to profilin (Phl p 12). Compared to other tested sera, this one recognised the greatest allergen number and most of the known allergens.

Serum #25 did not reveal Phl p 1b and 4 but recognised all others allergens revealed by serum #10 (data not shown). Serum #12 mainly recognised major allergens e.g. Phl p 1 and 5 (fig 1d). Allergen patterns of aqueous extract from non-exposed timothy

grass pollen, revealed by IgE from patient sera #10 and 25, were similar to these 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 to non-exposed pollen extract (Fig 1b).

2. Gaseous exposed pollen

2.1. Proteins from different pollen extracts

Timothy grass pollen grains were exposed to air, O_3, NO_2 or SO_2, alone or in combination. Using Bradford and Lowry methods, protein amounts in water soluble pollen extracts showed no significant difference between air-exposed and gaseous pollutant exposed pollens. By Bradford method, protein concentrations were 51.1 ± 3.7, 51.9 ± 3.6, 48.8 ± 8.0, $51.9 \pm 4.0\,\mu g$ of protein per mg of pollen, respectively for air-, O_3-, NO_2- and SO_2-exposed pollen extract.

2.2. 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).

2.3. 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 #10 (fig 2b), allergen Phl p 4 and Phl p 1b switched from pI 10.0 to 8.3. Similarly, the two remaining acidic spots corresponding to Phl p 5a and 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 kD, the patient serum #10 (fig 2b) revealed only an acidic spot around pI 3.2 corresponding to allergen [a]. Some of 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 to non-exposed pollen extract. Moreover Phl p 6 isoforms with pI 5.7-5.8 disappeared and in the Phl p 2 area (Mr 17-24 kD) a large decrease of isoform numbers was shown.

2.4.   2D allergen pattern of NO_2 exposed pollen

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

2.5.   2D allergen pattern of SO_2 exposed pollen

SO_2-treatment induced a decrease of IgE recognition of several allergens using the 3 sera. With serum #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 5 showed no significant difference. Whereas using serum #12, we observed a decrease in Phl p 1 and 5 recognition (data not shown).

2.6.   2D allergen pattern of gas mixture exposed-pollen

The allergen patterns obtained with serum #10 from combined gaseous pollutant exposed pollen extracts are shown on figure 3. A strong reduction of 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 results previously described were also obtained with sera #12 and 25 (data not shown).

In comparison with single pollutant exposed pollens (fig 2c and 2d), allergen pattern from NO_2/SO_2-exposed pollen (fig 3b) revealed a significant decrease of high Mr

allergens: [a], Phl p 4 and 13. The recognition of the other allergens was similar to the one obtained after SO_2 treatment (fig 2b).

Discussion:

When timothy grass pollen exposures were performed with realistic O_3 concentration or/and with NO_2 and SO_2 concentrations 10 times higher than the European warning level, we observed a decrease of some allergen recognition by IgE from three allergic patient sera. Acidification of several allergens was also observed following treatment using O_3 with or without NO_2. These experimental gaseous exposures didn't induce any apparition of *de novo* allergens. The decrease of allergen recognition suggests either the allergen loss during the treatment (for example the release in 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 pollutant concentrations than used by the former authors, inducing few damages on pollen grains and a weak intra-cytoplasmic granules 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 allergens 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 of the allergen recognition by IgEs. Goschnick *et al.*[33] analysed the effect of nitrogen dioxide (1-100 ppm) or ozone (0.1 ppm) treatment on three 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 v1, after exposition to a mixture of NO_2 (4.57 ppm) and O_3 (0.5 ppm). After exposure to this gas mixture, the level of protein nitration depends on the concentration of O_3 and NO_2 and their highly reactive species (NO_3 or HNO_3)[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 of the decrease of 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 (BN) 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 BN rats emphasize the difficulty to evaluate nowadays 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.

Figure legends:

Figure 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 (b): non-exposed, (c): air-exposed pollen revealed by IgE from grass pollen sensitive patient serum #10 and (d): non-exposed pollen revealed by serum #12. Each *P. pratense* allergens (Phl p) was surrounded and named (Allergome database) on the non-exposed blot (b). Isoelectric points (pI) (on the top) and relative molecular masses (Mr) (on the left) are indicated for each gel.

Figure 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 hours to 0.1 ppm O_3, 2 ppm of NO_2 or 2 ppm SO_2. (a): silver staining of proteins from O_3-exposed pollen. 2D immnnunoblots of (b): O_3-,(c): NO_2- and (d): SO_2-exposed pollen allergens revealed by IgE from grass pollen sensitive patient serum #10. Isoelectric points (pI) (on the top) and relative molecular masses (Mr) (on the left and right) are indicated for each gel.

Figure 3: 2D gel analysis of water-soluble extracts from NO_2/O_3- and NO_2/SO_2- exposed *P. pratense* pollen. Pollen grains were exposed during 4 hours to a mixture of either (a): 2 ppm NO_2 and 0.1 ppm O_3 or (b): 2 ppm NO_2 and 2 ppm SO_2. 2D immunoblots of (a): NO_2/O_3- and (b): NO_2/SO_2-exposed pollen allergens revealed by IgE from grass pollen sensitive patient serum #10. Isoelectric points (pI) (on the top) and relative molecular masses (Mr) (on the left and right) are indicated for each gel.

Acknowledgement

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Table I: Sources and comparison of European norms and doses used of gaseous pollutants (NO_2, O_3, SO_2)

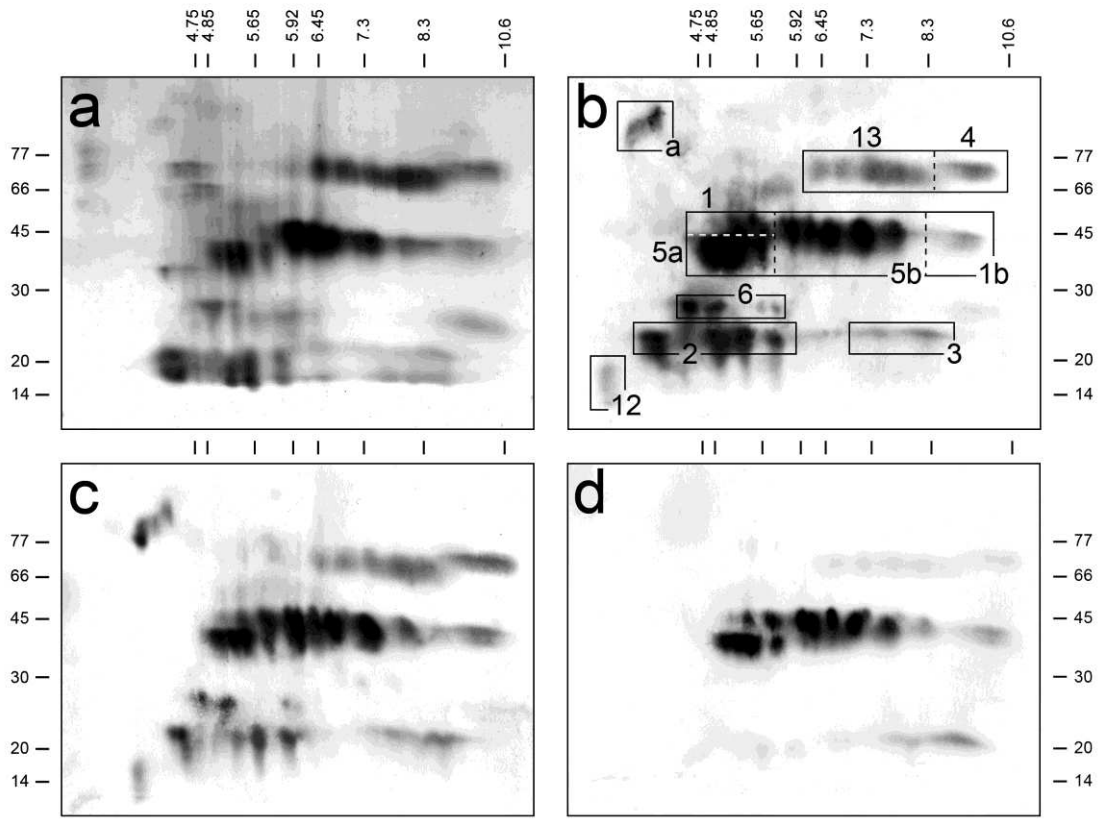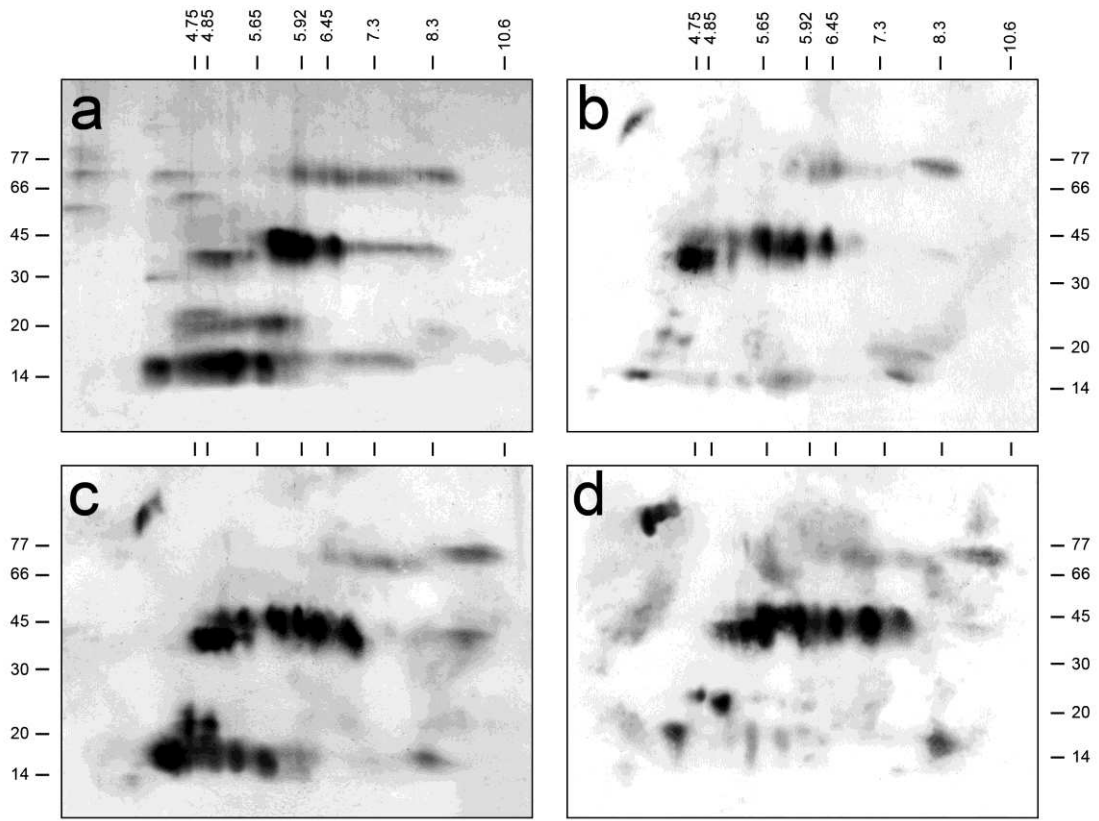| Pollutant	Source (main season concerned)	European norms	Doses used in the study
NO_2	Oxidation of NO emitting during high temperature combustion of fuels (power station and transport roads) ALL THE YEAR	52 µg/m^3 (average over year) Warning level : 400 µg/m^3 (3h)	2 ppm (4h) : 3800 µg/m^3
O_3	Secondary reaction between volatile organic compounds and NOx under UV SUMMER	120 µg/m^3 (average over 8 h) Information level : 180 µg/m^3 Warning level : 240 µg/m^3	0.1 ppm (4h) : 200 µg/m^3
SO_2	Combustion of fossil fuels containing sulphur (mainly from power stations) WINTER	20 µg/m^3 (average over year) Warning level : 500 µg/m^3 (3 h)	2 ppm (4h) : 5300 µg/m^3

Figure 1

Figure 2

Figure 3