

Evidence of grass-pollen allergenic activity in the smaller micronic atmospheric aerosol fraction

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Summary

In June 1988, during the grass-pollen season in Leiden, The Netherlands, outdoor airborne particulate matter was collected and separated into fractions according to aerodynamic sizes ($\geq 10 \mu\text{m}$, $4.9\text{--}10 \mu\text{m}$, $2.7\text{--}4.9 \mu\text{m}$, $1.3\text{--}2.7 \mu\text{m}$, $0.6\text{--}1.3 \mu\text{m}$, $\leq 0.6 \mu\text{m}$), with a cascade impactor mounted on top of a high volume sampler. The different fractions were tested for the presence of grass-pollen allergenic activity using a RAST-inhibition assay: specific IgE-antibody-containing patient serum was applied on the particle-loaded impaction strips, and the serum was recovered by descending elution for further analysis in the RAST. Simultaneously, continuous measurements were made of the airborne grass-pollen concentration using a volumetric pollen trap. Sampling observations lasting 7–9 hr during a period with relatively high airborne grass-pollen concentrations showed reliably detectable amounts of grass-pollen allergen, not only in the first impaction stage where intact pollen were collected, but also in the lower stages collecting the smaller, paucimicronic and submicron atmospheric aerosol fraction. It is evident that this result has serious implications for the understanding of the bronchial symptoms frequently seen in hay fever patients on days with high pollen concentrations in the air.

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Introduction

Airborne allergenic activity is usually thought to be carried by or associated with relatively large and often morphologically identifiable particles floating in the air. Obvious examples of this association are pollen grains, mould spores, animal skin scales, and mite faecal pellets, mostly particles with sizes between 3 and $50 \mu\text{m}$. Aerodynamic properties of these particles, related to size, form, and density, are of importance for the region in the respiratory tract where the particles will be deposited, thereby determining the target organ in which the first allergic reaction manifests itself in sensitive subjects.

However, the allergic symptoms are very often not limited to the region where the particles are supposed to be deposited. The best known of these controversies exists in relation to pollen allergies. In fact, Blackley [1] has

already speculated about 'free granular matter' that floats in the air carrying the substance causing 'hay-asthma'. Normally, intact pollen grains, with sizes of $15\text{--}40 \mu\text{m}$, should impact on the nasal and nasopharyngeal mucus membranes [2–5] giving rise to the typical symptoms of pollinosis. However, symptoms of the lower bronchial regions of the respiratory tract, like coughing and wheezing, are also frequently seen in pollen allergic patients. Although it is plausible that a small number of airborne pollen grains might be capable of penetrating into the lower regions of the respiratory organs [6–7], suggestions and evidence for airborne allergenic activity associated with particles smaller than intact pollen grains have been put forward by several authors [8–16].

One of the methodological keystones to demonstrate allergenic activity on particles of small sizes is the sampling in different fractions according to the aerodynamic sizes of the particles. Various techniques of fractionated aerodynamic sampling, in both outdoor and indoor situations, combined with different immuno-

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chemical identification methods, have been employed to measure airborne concentrations of small particles carrying allergenic activity originating from ragweed pollen [12], birch pollen [16], dust mite faeces [17,18], and mammalian products [17–21]. Suggestions for the presence of airborne grass-pollen allergen outside the pollen grains have been put forward by Stewart and Holt [13], Schumacher *et al.* [14], and Jensen *et al.* [15]. However, sampling of multiple size fractions followed by immunoassay assessment of airborne grass-pollen allergenic activity has not yet been reported.

In this study a high volume sampler fitted with a cascade impactor was used, essentially as described by Agarwal *et al.* [12]. Airborne allergen, captured on multiple impaction sheets, was measured by RAST-inhibition by application of grass-pollen specific IgE antibodies and subsequent recovery of the remaining free antibodies by descending elution, followed by analysis of antibody presence in the eluate by the RAST.

Materials and methods

Particle sampling

Location of the air samplers was on the flat roof of a five-storey building of the Leiden University Hospital, in the western part of the town. These were installed approximately 2 m apart.

Observations of airborne pollen concentrations were made with the volumetric Burkard® (Richmansworth, U.K.) pollen trap, as described by Leuschner [22]. The Burkard trap was operated continuously.

For the sampling of airborne allergen, a high volume sampler, GMWS-2310 (General Metal Works Inc., Cleves, U.S.A.) was used [23]. The volume of the airflow (capacity ranging from 0.6 to 1.6 m³ min⁻¹) was set at 1.13 m³ min⁻¹, and was automatically adjusted for variations in the flow rate, *e.g.* caused by loading of the filter. The high volume sampler was operated for 1–3 hr for short sampling observations, and 7–9 hr for long observations.

On top of the high volume sampler, a five-stage cascade impactor, Sierra, model 235 (Andersen Samplers Inc., Atlanta, U.S.A.) was mounted. This cascade impactor consisted of five aluminium plates (15.2 × 17.8 cm) provided with ten (the uppermost plate with nine) 12.3-cm long slot-formed air passages of decreasing widths, namely 4.0, 1.5, 1.0, 0.5, and 0.3 mm, respectively. This decreasing slot width causes an increase of the velocity of the air stream. From top to bottom, the slots have alternating positions allowing particles to impact on strips of collector sheets underneath the slot, depending on aerodynamic mass (inertia) of the particles and the speed of the air flow. Thus, five fractions of different particle sizes can be separated and collected on impaction strips.

At a flow rate of 1.13 m³ min⁻¹, particles with a diameter of ≥ 10 µm will be collected on stage I of the impactor. The particle size cut-offs for 50% collecting efficiency (for spherical particles with unit mass density) at the four lower impaction stages are, II: 7.2 µm; III: 3.0 µm; IV: 1.5 µm; V: 0.95 µm, respectively, according to the specifications of the manufacturer. These 50% cut-off efficiencies correspond with the following size ranges, comprising approximately 80% mass of the size fraction particles: II: 4.9–10 µm; III: 2.7–4.9 µm; IV: 1.3–2.7 µm; V: 0.6–1.3 µm.

Underneath the nine or ten air-passage slots in each of the five plates of the cascade stages, strips of impaction sheets were fitted, approximately 1-cm wide and 20-cm long, leaving one end blank for 1 cm and the opposite end for almost 7 cm. At the bottom of the impactor there was a back-up filter (20 × 25 cm; effective filtering area: 17 × 22 cm) collecting the particles smaller than those captured by the last impaction stage, *i.e.* ≤ 0.6 µm. After a sampling observation, this back-up filter was cut into 22 strips of similar dimensions to the impaction strips. Consequently, each impaction strip was carrying 1/10 (the uppermost 1/9) of the total fraction's quantity, each back-up filter strip only 1/22.

In summary, a sampling observation resulted in five sheets of ten (the uppermost of nine) 20-cm long impaction strips with a 7-cm long blank end and a 12.3-cm long narrow band of deposited particles of decreasing size ranges, and one back-up filter evenly loaded with the smallest particles, to be cut into strips of similar form.

In these experiments, impaction sheets of glass fibre TF AGF810 (Staplex, Brooklyn, New York, U.S.A.) were used cut into strips of dimensions described above. According to the specifications of the manufacturer, this type of filter had a more than 99% filtering efficiency for particles as small as 0.3 µm, applicable to the back-up filter.

To verify the sizing efficiency of the cascade impactor, pieces of 1 cm from one strip of each of the five impaction stages and an equally sized part of the back-up filter, selected at random, were examined with a Cambridge S 180 scanning electron microscope at an accelerating voltage of 15 kV, and a tilting angle of about 30° relative to the electron beam [24]. Before examination, the preparations were covered with a layer of gold about 20 nm thick [24], using a Bolzers Sputter-coater (Bolzers Union AG, Lichtenstein). Bands selected at random were inspected at a magnification of × 500, and areas selected at random were photographed at a magnification of × 1440.

Application of specific IgE antibodies to strips and elution

Detection of allergenic activity was established by blocking of human anti-grass-pollen IgE antibodies and sub-

sequent measuring of remaining specific IgE reactivity in the RAST ('RAST-inhibition') [25]. Antibodies were obtained from a pool of sera from eight hay fever patients with a class 3-positive RAST result with *Dactylis glomerata* (cocksfoot) pollen allergen (G3; Pharmacia Diagnostics AB, Uppsala, Sweden).

Following air sampling, the particle-loaded impaction strips were moistened with approximately 400 μ l of phosphate-buffered saline (PBS) to release the allergen from the particulate matter. After the strips had been dried overnight, 100 μ l of the specific IgE-containing serum pool, diluted 2- or 10-fold in RAST buffer (0.1 M phosphate, 0.2% bovine serum albumin, 1% Tween-20, 0.01% azide; pH 7.5), were applied on the 7-cm long blank end of the strip just above the band of deposited particles. This end of the strip was then mounted in a buffer reservoir and the opposite smaller blank end of the strip, which was cut so that it tapered to a point, was placed downward just above a small 1.5-ml polypropylene vial. Descending elution was performed using RAST buffer until 0.5 ml eluate was collected, which took at least 60 min. This elution procedure allowed the applied specific IgE antibodies to react with the allergen on the impaction strip. The remaining unbound specific antibodies and (part of) the allergen-antibody complexes were collected in the eluate.

Measurement of allergenic activity

The allergenic activity was determined by measuring the remaining amount of free specific IgE antibodies in the eluate obtained as described above, relative to the amount of specific IgE antibodies in an eluate obtained by elution of a blank strip on which the specific IgE-containing serum pool dilution was applied. The specific IgE antibodies were measured with commercially available reagents (Pharmacia Diagnostics AB) as follows: to the eluate (0.5 ml), a grass mix-loaded disc ($G \times 1$) was added and incubated overnight at room temperature while rotating the tube. After the disc was washed, the subsequent steps of the test were performed as described by the manufacturer of the RAST kit. The relative reduction in IgE-antibody activity, which is proportional to the amount of IgE-antibody activity blocked by allergen, is expressed as a percentage inhibition, calculated as follows:

$$\frac{\text{counts in eluate from allergen-free strip} - \text{counts in eluate from allergen-carrying strip with grass-pollen negative serum}}{\text{counts in eluate from allergen-carrying strip}} \times 100$$

Non-specific binding of human IgE antibodies to the strips was controlled by application and elution of serum from cat-allergic patients and subsequent measurement of cat-specific IgE antibodies in the eluate. The percentage non-specific loss ranged from 15 to 25, and was always similar when blank filters or particulate-loaded filters were used.

Model experiment with applied pollen extract

In order to check the complete procedure of the various steps and elements of the elution and RAST-inhibition techniques, a model laboratory experiment was carried out by applying different dilutions of *D. glomerata* pollen extract on blank strips. Distributed in 12 aliquots over the length of the strip, 250 μ l extract were placed in concentrations corresponding to an absolute amount of 0, 3, 10, 30 and 100 μ g dry *D. glomerata* pollen per strip, respectively.

According to Brown and Irving [26], 68 pollen grains of *D. glomerata* constitute 1 μ g, so the amount of allergen applied to the strip would come from 0, 204, 680, 2040, and 6800 pollen grains per strip, or 0, 1836, 6120, 18 360, and 61 200 per upper impaction sheet of nine strips.

After drying the strips overnight, 100 μ l of 10-fold diluted serum were applied as described above. On two control strips with extract of 0–100 μ g pollen, RAST-negative serum was placed. Directly following serum application, the strips were eluted as described above, and in the 500 μ l eluates the relative amount of grass-pollen-specific IgE was measured by RAST. Percentage inhibition compared to the eluate from the strip without allergen was calculated, again as described above. Loss of IgE due to non-specific binding to the strip was controlled by including RAST-assays without strips.

Results

Model experiment with applied pollen extract

During a dry summer's day, an outdoor airborne pollen concentration of 50/m³ is realistic. With the sampler set at an air flow rate of 1.13 m³ min⁻¹, and assuming that intact grass-pollen grains (20–30 μ m) would be collected in the first stage of the cascade impactor, then the sampling times to collect the amount of allergen corresponding to the five quantities applied for the model experiment (see Materials and methods) would be 0, 0.5, 1.8, 5.4, and 18.1 hr, respectively.

The results of this model recovery experiment show that, within the expected range of sampling times and airborne grass-pollen allergenic activity, RAST inhibition, carried out as described, can be accomplished successfully. A linear relationship was found between the percentage of inhibition in the RAST and the log concentration of the applied grass-pollen extracts (Fig. 1).

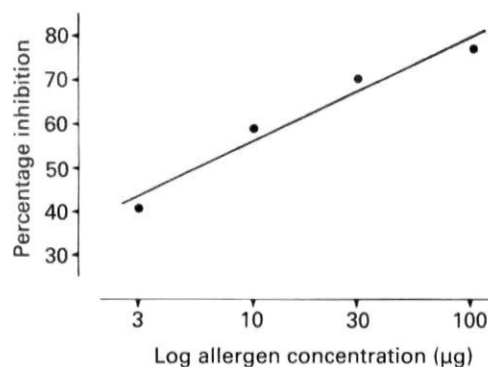


Fig. 1. Percentage of inhibition in the RAST measured in a pool of human serum (diluted 10-fold), containing specific anti-*D. glomerata* pollen IgE antibodies, which was eluted over strips of glass fibre sheets on which increasing amounts of *D. glomerata* pollen allergen extract had been applied. Calculations were performed according to the formula given in Materials and methods.

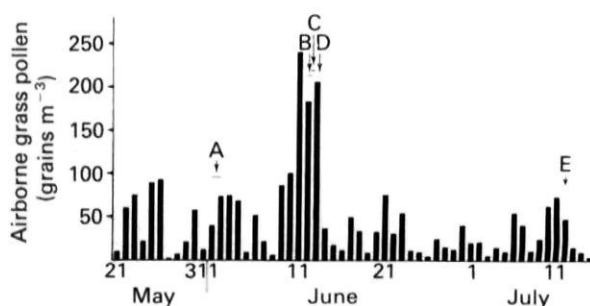


Fig. 2. Daily fluctuations in the airborne grass-pollen concentration in the summer of 1988, measured at Leiden with a volumetric spore trap (Burkard®). Five cascade-impactor samplings are indicated (A-E).

Seasonal airborne grass-pollen concentration in 1988

The seasonal course of the airborne grass-pollen concentration varies from year to year [27]. Therefore, timing of air sampling for measurement of grass-pollen allergenic activity in relation to grass-pollen concentrations in the air should be planned and evaluated using a knowledge of these variations. Seasonal grass-pollen concentrations during the 1988 season are shown in Fig. 2, in which the dates of fractionated sampling for allergenic activity are also indicated (A-E).

From 1 to 2 June, in the beginning of the season, a 24-hr pilot sampling (A) was carried out as a final test of the functioning of the cascade impactor and the high volume sampler on the roof of the hospital building, and of the elution and inhibition procedure in the laboratory. No data of collected allergenic activity are shown.

Two observational samplings were carried out in two subsequent periods of 9½ and 7 hr on 12 June (B), and from 12 to 13 June (C), respectively. As can be seen in Fig. 2, these samplings took place during the only short peak period of the airborne grass-pollen concentration course in the damp and cool summer of 1988 at Leiden.

Two more short-time sampling of approximately 1 and 3 hr were carried out on 13 June (D), and on 12 July (E).

Efficiency of particle size separation by the cascade impactor

After the sampling on 12 June (B; Fig. 2) 1-cm long randomly selected pieces of a strip from each of the five impaction plates, and an equally sized part of the back-up (bottom) filter, were carefully and systematically inspected by scanning electron microscopy. No attempt was made to quantify the number of pollen grains in the inspected area, but, qualitatively, in impaction stage I several grains of wild grasses (Poaceae) and of elder (*Sambucus*) were seen. In stage II only one pollen grain could be found, while in stages III, IV and V, and on the back-up filter no pollen grains were seen. Of each of the six filter pieces, five randomly selected areas were photographed, of which typical examples are shown in Fig. 3. Although there was some particle-size overlap, probably due to aberrations in aerodynamic mass, it can be seen that there is effective fractionation of the particles over the six impaction stages according to size. Identifiable biological particles are the grass-pollen grain on the strip of stage I, and several conidia ('spores') of the mould *Cladosporium* on the strips of stages II and III.

Allergic activity in the different size fractions

The relative allergenic activity, as collected from the air on the different impaction stages and the back-up filter, was measured after two observational samplings during a sunny and dry weather period in the middle of June (Fig. 2).

The first sampling (B) was carried out on 12 June, from 1245 to 2220 hr local summer-time. It was calculated, based on the airflow of 1.13 m³ min⁻¹ of the sampler, that a volume of 650 m³ of air was drawn through the cascade impactor during this first sampling period. The average number of grass-pollen grains during that period was 155 m⁻³, so that approximately 100 750 grains may be assumed to be sampled and collected on the top plates of the impactor. Maximum daytime temperature was approximately 20°C, relative air humidity was 50–60%, and a moderate wind was blowing from NE to E, i.e. from an inland direction. Relative allergenic activities on the strips of the different impaction stages (I–V) and on the

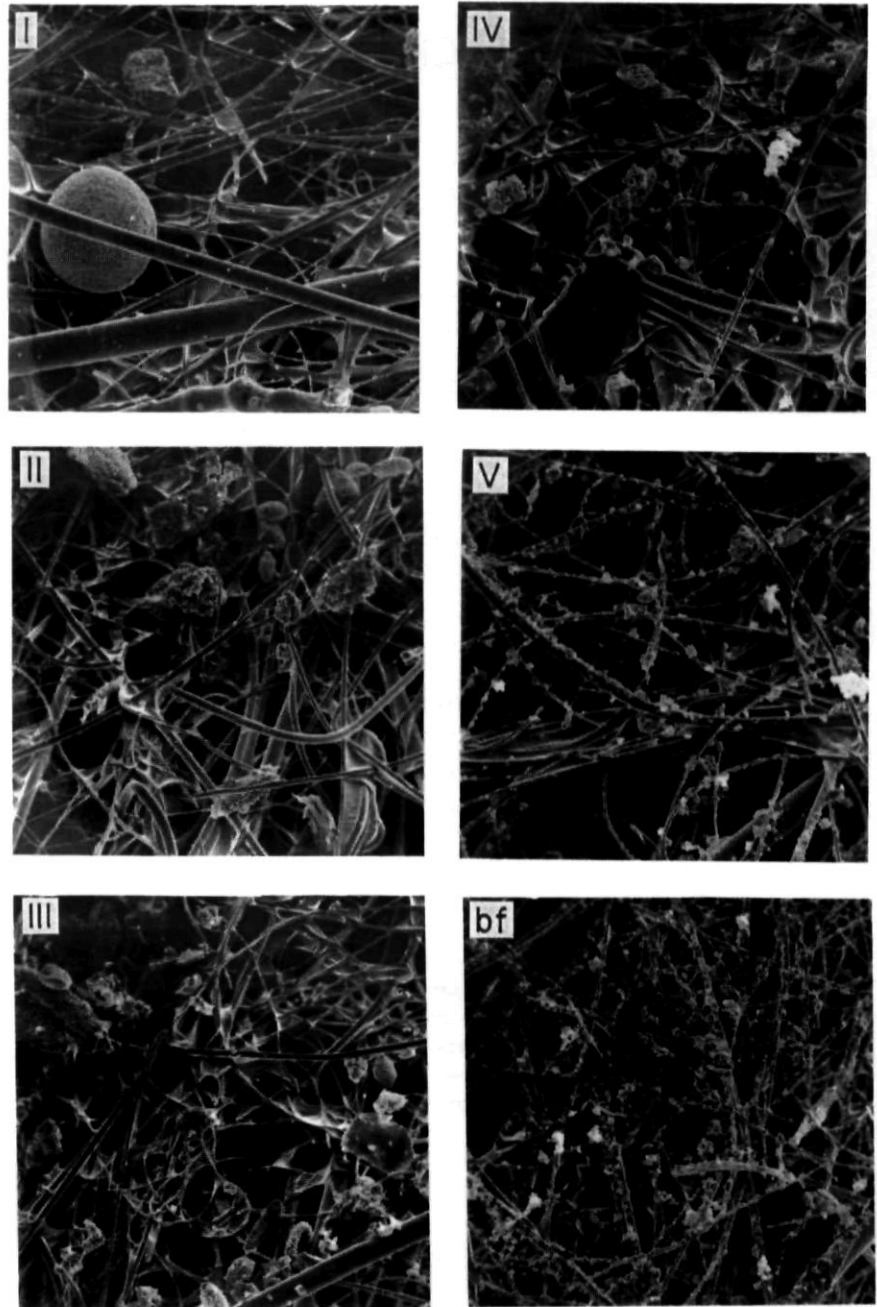


Fig. 3. SEM photographs ($\times 1368$) of randomly selected areas of five cascade-impactor stages (I–V) and the back-up (bottom) filter (bf), showing particles of different sizes. On the sheet from stage I, which is almost clean, a grass-pollen grain is visible; on the sheets of stages II and III various conidia and spores of moulds (e.g. *Cladosporium*) are recognizable. The bottom filter carries a high amount of unidentifiable ‘amorphous’ dust particles of micronic sizes.

back-up (bottom) filter were measured by RAST-inhibition after application of twofold-diluted RAST-positive serum and subsequent elution, as described. Results are given in Table 1 and Figure 4.

The second observational sampling (C; Fig. 2) took place in the evening and night of 12–13 June, from 2250 to 0555 hr, thus during 7 hr and 5 mins. Again with the sampler’s flow of $1.13 \text{ m}^3 \text{ min}^{-1}$, a volume of 480 m^3 of air was drawn through the impactor, carrying $312 \text{ pollen grains m}^{-3}$ (approximately double the day’s average),

resulting in approximately 149 750 grains being captured by the impactor’s top stages. The minimum temperature that night was approximately 10°C , relative air humidity between 60 and 70%, still with a moderate NE–E overland wind. Table 1 and Fig. 4 show the relative grass-pollen allergenic activities on the strips of the five impaction stages and the back-up (bottom) filter, determined as described previously.

On 13 June and on 12 July two short samplings (D and E; Fig. 2) were carried out with the following character-

Table 1. Air volume, pollen concentration and relative grass-pollen allergenic activities in different particle size fractions, expressed as percentages of RAST-inhibition* of two sampling periods

Date of sampling (1988)	Volume of air (m ³)	Number of grass-pollen grains	Rast-inhibition percentages in different size fractions (µm)					
			Impaction stages					Back-up (bottom) filter ≤0.6
			I ≥ 10	II 4.9–10	III 2.7–4.9	IV 1.3–2.7	V 0.6–1.33	
12 June (B)	650	100 750	37.8	40.3	25.6	35.2	36.8	63.0
12/13 June (C)	480	149 750	44.8	56.7	47.9	48.5	45.4	65.0

* RAST-inhibition experiments were performed using a pool of twofold diluted *D. glomerata* pollen-positive sera. The inhibition percentage was calculated relative to RAST results obtained with serum eluted from allergen-free blank strips according to the formulas given in Materials and methods.

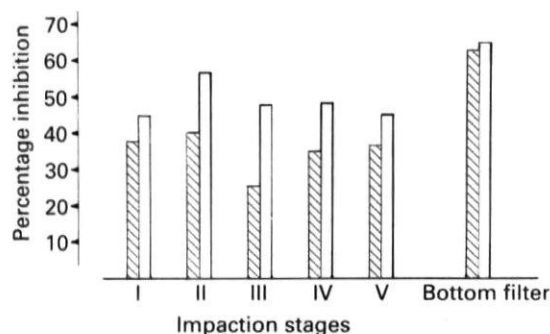


Fig. 4. Relative grass-pollen allergenic activity in different particle-size fractions, expressed as percentages of RAST-inhibition (see also Table 1). Allergenic activity is found in all impaction stages, and also on the back-up (bottom) filter. (□) First observational sampling, daytime, 12 June (B; Fig. 2). (▨) Second observational sampling, night-time 12–13 June C; (Fig. 2).

istics. June 13: duration 65 min, airflow 1.07 m³ min⁻¹, airborne pollen concentration 1.55 m⁻³, estimated number of sampled grass-pollen grains 10 800. July 12: duration 175 min, airflow 1.16 m³ min⁻¹, airborne pollen concentration 66 m⁻³, estimated number of sampled grass-pollen grains 13 400. The impaction strips obtained from these two samplings were tested for relative grass-pollen allergenic activity with 10-fold, instead of twofold, diluted RAST-positive pooled serum. Notwithstanding this adjustment in the serum solution, the results of the RAST-inhibition assay were under 25%, too low for a reliable interpretation, as can be deduced from the results shown in Fig. 1. These results are therefore not taken into consideration.

Discussion

The primary aim of this study was to investigate the presence of grass-pollen allergenic activity in the outdoor air associated with particles of size fractions much smaller than intact pollen grains. Such presence has been reported before for ragweed by Agarwal *et al.* [12], and for birch in a preliminary report by Rantio Lehtimäki *et al.* [16], using similar or comparable impaction sampling techniques to those employed in this study. Confirming results obtained with filtration (rather than impaction) are reported for ragweed by Habenicht *et al.* [11], and, in a pilot study, for rye grass by Stewart and Holt [13]. The results presented here, using a cascade impactor for the fractionated sampling of smaller micronic fractions, strongly suggest the existence of airborne grass-pollen allergens associated with these particle-size fractions, as shown by the clear and undeniable RAST-inhibition data obtained with exposed impaction strips from all stages of the cascade impactor and of the back-up (bottom) filter.

The efficiency of the cascade impactor, as claimed by the manufacturer, seems to be achieved sufficiently well, as illustrated by the size distribution of the impacted particles shown in the SEM-photographs (Fig. 3). The impaction stages I–V are collecting particles of rather well-defined size fractions. However, the relative quantities or mass of the different fractions of the collected material is completely unknown. Observing the strips with the naked eye, one can see the impacted material as a very light yellow-grey band on stage I (where most of the pollen grains are), becoming darker and narrower on every lower stage, and ending as a sharp dark grey line in stage V carrying particles measuring approximately 1 µm. The back-up filter, through which all the air is drawn, carries the submicron particles, and is uniformly dark

grey after 7–9 hr exposure. It seems as if this back-up filter is collecting the bulk of the airborne particulate matter, confirming the known phenomenon that, both numerically and in mass, the majority of airborne particles are of submicron dimensions [10,23].

The allergen detection method used in this and other studies [12,15] is not very sensitive and requires sampling of relatively high quantities of air. This is particularly true for the collection of most outdoor airborne allergens like that of pollen, the concentration of which is generally rather low compared to short peak concentrations that sometimes occur indoors, e.g. with rat antigens in animal laboratory environments [21]. In the experiments described here, a volume of at least 500 m³ of air on a day with favourable weather during the grass-pollen season appears to be a minimal quantity, which, with this type of equipment, requires a sampling period of approximately 8 hr. This period is longer than that used by Price and Longbottom [21], who sampled laboratory animal allergens during 1 hr, but less than that used by Agarwal *et al.* [12], and by Jensen *et al.* [15], who sampled for 24 hr to collect ragweed, and timothy and birch pollen allergen, respectively.

It is often believed that sampling large volumes of air also means greater velocities of the air flow [18], causing pollen grains either to undergo fragmentation or ricocheting in the impactor to lower stages. However, effective separation of particles into size fractions by impactor stages needs different but rather well-defined air speeds at the various stages, regardless of the volumes involved. This has been confirmed by comparative calculations on the real air speed for two different cascade impactors. For instance, the air velocities in the four small jets in the Casella cascade impactor, as used by Platts-Mills *et al.* [18] and by Price and Longbottom [21], are 2.2, 10.2, 27.5, and 77 m sec⁻¹ [28]. The airflow speeds in the long slots of the stages of the Sierra cascade impactor, mounted onto a high volume sampler operating at a capacity of 1.13 m³ min⁻¹, as used in this study, are calculated to be 4.3, 10.2, 15.3, 30.6, and 51.0 m sec⁻¹, respectively. Thus there is no reason to think that collecting particles with a high volume sampler leads to more violent effects on pollen grains than sampling with smaller devices. Moreover, inspection by SEM did not show any traces of pollen-grain fragments.

Accepting the fact that there is a substantial amount of airborne grass-pollen allergenic activity associated with particles smaller than pollen grains, the question arises whether a relationship exists between the airborne allergen levels and the airborne pollen concentration. Although from the very limited number of observations in this study hardly any conclusions can be drawn, the results suggest that the air sample with the higher pollen

concentration (12–13 June) also gave relatively stronger RAST-inhibition compared to the air sample with the lower pollen count (12 June). Also, Jensen *et al.* [15] found a relationship between pollen count and allergen quantity in the air, contrary to Schumacher *et al.* [14] and Stewart and Holt [13] who did not find such a relationship. It is evident that more observations are required to answer this question explicitly.

With regard to the nature and origin of the paucimicronic and submicron atmospheric aerosol fractions, carrying pollen-allergenic activity, no observational data are available. However, it is evident that their atmospheric occurrence is of great importance for the understanding of allergic reactions in the lower region of the respiratory tract, often seen in pollinosis.

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