

Airborne birch pollen antigens in different particle sizes

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Summary

Two particle samplers for ambient air, situated together: a static size-selective bio-aerosol sampler (SSBAS) and a Burkard pollen and spore trap were compared in sampling intact birch pollen grains through one flowering period of *Betula* (a total of 44 days). The SSBAS trapped pollen grains three times more efficiently than the Burkard trap, but the variations in pollen counts were significantly correlated. In contrast, birch pollen antigenic activity and the pollen count in the Burkard samples were not closely correlated. The antigenic concentration was occasionally high both before and after the pollination period. There was a high birch pollen antigenic activity in particle size classes where intact pollen grains were absent, even on days when the pollen count was very low. Correspondingly, on days with high birch pollen counts in the air, pollen antigenic activity was on several occasions low, indicating that pollen grains were empty of antigenic material. The small particle size classes are especially important to allergic patients because they are able to penetrate immediately into the alveoli and provoke asthmatic reactions. Therefore, aerobiological information systems based on pollen and spore counts should be supplemented with information concerning antigenic activities in the air.

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Introduction

As long as aerobiological samples have been taken, one of the main principles has been to simulate human inhalation as far as possible both in volume and in particle fractionation according to aerodynamic size [e.g. 1,2,3]. Aerodynamic properties are important in the analysis of particle deposition in the human respiratory tract, assuming allergic symptoms to appear first in the target organ [4,5]. Intact pollen grains, due to their size 10–80 μm in diameter, should be retained in the upper airways (the nasal and nasopharyngeal mucous membranes). However, persons allergic to pollen often show symptoms of asthma, indicating the presence of antigens similar to pollen antigens in size fractions smaller than intact pollen grains. A number of authors have documented antigenic activity in small particle size classes [6–14]. This phenomenon presents new demands to aerobiologists, not only to count particles in samples but to develop new (immuno) chemical methods for the analysis of the actual antigen concentration in the air. In taking samples for these

purposes, most authors [e.g. 6,13,15] recommend high volume samplers because they guarantee an air volume large enough to detect the allergenic activity (e.g. with RAST inhibition methods). Our aim was to find a method of sampling in which simulation of human respiratory tract in particle fractionation would be borne in mind, and to develop a sensitive ELISA (enzyme-linked immunosorbent assay) modification to determine the antigenic activity of the samples.

Thus, the aims of this study were: (a) to compare the newly developed SSBAS sampler with the continuously operating Burkard pollen and spore trap in routine aerobiological information service; (b) to compare airborne birch pollen counts to birch pollen antigenic activity in different aerodynamic particle size classes, and (c) to evaluate the difficulty of replacing the present aerobiological information system with reports on antigen concentrations in the air.

Materials and methods

The two pollen and spore samplers tested, the volumetric

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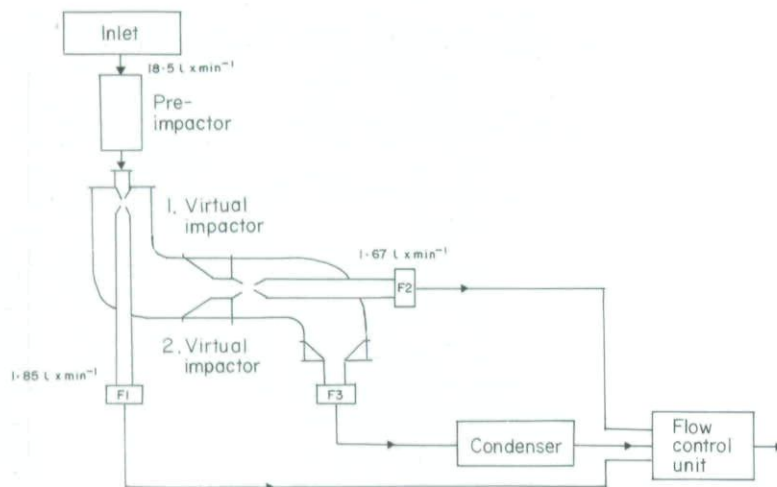


Fig. 1. Schematic diagram of the SSBAS with air flow rates in three filter stages (F_i ; $i = 1, 2, 3$). The pore size of the filter used (Millipore membrane filters) was $0.7 \mu\text{m}$ in stage 1 and $0.45 \mu\text{m}$ in stages 2 and 3. The sample in the condenser unit (-20°C) is liquid when thawed.

Burkard trap (Rickmansworth, UK; a modified version of Hirst's spore trap [2] and the size-selective bio-aerosol sampler (SSBAS) were situated together 15 m above ground level on the roof of a university building in Turku. The SSBAS fractionates airborne particles in size classes as follows: filter stage 1: $> 7.2 \mu\text{m}$; 2: $7.2\text{--}2.4 \mu\text{m}$ and 3: $< 2.4 \mu\text{m}$ in aerodynamic diameter. Sample 4 (submicronic antigen-bearing particles) is collected in a liquid-cooled (-20°C) condenser unit. The design and calibration procedures of the SSBAS have been described in detail by Kauppinen *et al.* [16] (see also Fig. 1). Microscopic particle counts to compare the sampling efficiencies of these two traps were performed in 1985. Particle counts were analysed for four squares (approximately 1.4 cm^2) of each gridded filter (Nuclepore Membra-Fil), cleared with immersion oil. These results were compared with daily Burkard counts.

Air samples for the analysis of antigenic activity of birch pollen were taken daily in 1989, from 20 April to 21 June, excluding a rainy period between 3 and 8 June. The filters (Millipore, type HA), changed daily, were sealed in a sterile petri dish and stored at -50°C until analysed. The sample in stage 4 was first thawed because it condenses in a jacketed condenser, then refrozen on the walls of a round-bottomed flask in a thin layer. The sample was then lyophilized and stored at -50°C in a glass tube. The filter discs for the ELISA procedure were obtained by cutting the filters from stages 1, 2 and 3 by a 5 mm cork borer. One disc represented 1:74 of the total effective sampling area on the filter. The filter discs were immersed in $125 \mu\text{l}$ phosphate-buffered saline (PBS, pH 7.4) in E.I.A./R.I.A. 96-well microtitre plates (Costar Co.) and incubated overnight at 37°C to allow released birch pollen antigens to bind to the solid phase. The filter discs were then collected and the microtitre plates washed three times with PBS ($200 \mu\text{l}$ each), incubated in $125 \mu\text{l}$

PBS-BSA-Tw (containing 0.5% BSA + 0.5% Tween 40 to prevent non-specific binding of antibodies) 1 h at 37°C , and again washed twice with PBS. The lyophilized samples from the condenser unit (stage 4) were dissolved in 1.5 ml PBS, divided into $125 \mu\text{l}$ aliquots (four duplicates) and incubated overnight at 37°C similarly to the filter discs. The bound birch antigen was detected using $125 \mu\text{l}/\text{well}$ antibodies against *Betula* pollen produced in rabbit (diluted in 1:1000 in PBS-BSA-Tw; ALK A/S, Copenhagen). Normal rabbit serum was used as a control. After 2 h incubation at 37°C the plates were washed twice with PBS. Thereafter HRP (horseradish peroxidase)-conjugated swine antibodies to rabbit immunoglobulins (DAKO A/S) ($100 \mu\text{l}/\text{well}$ of 1:1000 dilution in PBS-BSA-Tw) was added and incubated overnight at RT. After three washes with PBS the enzyme activity of the bound anti-rabbit-IgG-HRP conjugate was determined by adding o-phenyldiamine substrate diluted in citrate buffer (45 mg o-phenyldiamine in 15 ml citrate buffer pH 5.5 + $10 \mu\text{l}$ H_2O_2) $75 \mu\text{l}/\text{well}$, incubated at RT for about 30 min (colour development was followed by eye). The reaction was stopped by adding $125 \mu\text{l}$ of 1 N HCl. The optical absorbance was measured at 492 nm by Multiscan® microplate reader (Flow Lab. Inc.). All filter samples were analysed in four duplicates. Control filter discs of both pore sizes (0.7 and $0.45 \mu\text{m}$) were used for correcting the absorbances (as blanks). The birch pollen extract used for standards was *B. verrucosa* extract of $100\,000 \text{ SQ-units/ml}$ (ALK A/S, Copenhagen). Dilutions of 1:5000, 1:10 000, 1:100 000 and 1:500 000 of birch pollen extracts were used. The antigenic activities of each stage were transformed to correspond to $\text{SQ-units} \times \text{m}^{-3}$ of air using the intake air volume measured in the flow control unit of the SSBAS. Correlations between the four stages and with the Burkard results were counted using the CORR procedure of SAS statistics [17].

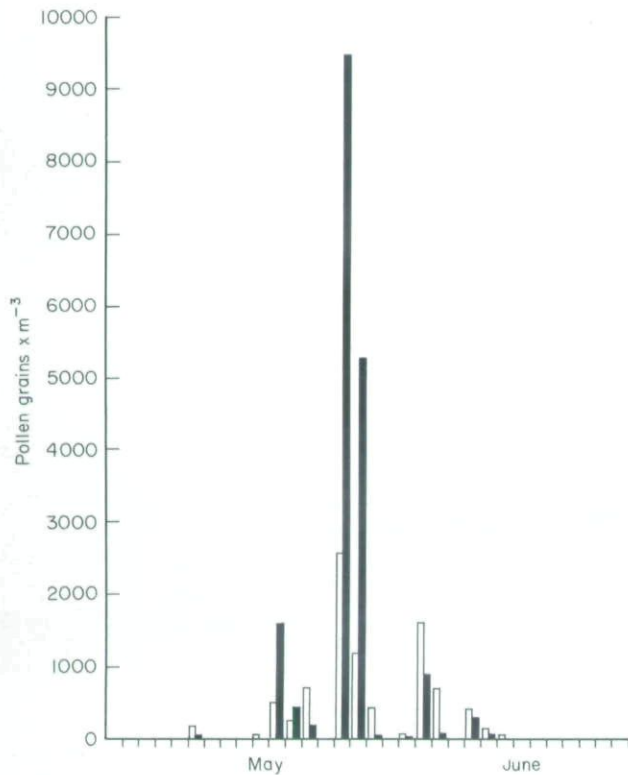


Fig. 2. Burkard (□) vs SSBAS (■); *Betula* pollen counts (microscopical) in Turku, 1985.

Results

The frequencies of intact *Betula* pollen were three times higher in SSBAS filter stage 1 than in the Burkard samples (Fig. 2). This indicates that the SSBAS is efficient also in the size class of *Betula* pollen (approximately 23 µm in

Table 1. Correlation matrix of four SSBAS stages (birch antigenic activities) and birch pollen counts in Burkard samples during the birch pollen season 1989, in Turku.

	F2	F3	F4	Burkard
F1	0.1027 (<i>P</i> =0.507)	0.1774 (<i>P</i> =0.249)	-0.092 (<i>P</i> =0.553)	0.2914 (<i>P</i> =0.055)*
F2		0.532 (<i>P</i> =0.000)***	-0.0607 (<i>P</i> =0.696)	-0.0399 (<i>P</i> =0.797)
F3			0.1051 (<i>P</i> =0.497)	0.0714 (<i>P</i> =0.645)
F4				-0.0408 (<i>P</i> =0.793)

diameter). It is possible that birch pollen counts are underestimated in Burkard counts in general. Only two single pollen grains were detected in filter stage 2, and none in stages 3 and 4 during the whole birch pollen period for 1985. This was only 0.01% of the total pollen count. The daily pollen frequencies in the two samplers were significantly correlated ($R=0.8448$, $P<0.055$). This suggests that some grains were probably empty and thus without antigenic activity.

Birch antigen activities (1989) were significantly inter-correlated in stages 2 and 3 (Table 1). The activity in stage 4 had a tendency to increase towards the end of the pollen season after one week's rainy period, maximum value occurring on a day with a low pollen count (Fig. 3). Taking the birch pollen season for 1989 as a whole, 65.5% of the total antigenic activity was found in stage 1, 4.7% in stage 2, 10.4% in stage 3 and 19.3% in stage 4; thus 34.5% of the total antigenic activity of birch pollen was detected in stages with no intact pollen grains. In the ELISA analyses a detection limit of 0.300 SQ units was used. Maximum variation accepted in quadruplicates of the filter discs was within the same limits. All four SSBAS stages produced ELISA dose-response curves with slopes that did not differ significantly from that of the *B. verrucosa* standard.

Discussion

Birch pollen grains are known to be distributed very evenly in air in a vertical direction [18]. We know, however, nothing about the distribution of small micro-nic particles of birch antigens, but due to their small size they are expected to occur representatively at 15 m elevation. Spieksma *et al.* [13], using a cascade impactor mounted on top of a high volume sampler, report that this construction does not fragment pollen grains even at high air velocities; the air speeds at various stages merely have to be well-defined. However, small particles may be released from pollen grains in the first stages and then transported to lower stages when high flow rates are used. We prefer to use a sampler with a low air intake value. In the SSBAS, conduction is impossible because the incoming air does not pass successive filter stages but is divided into two separate air flows, and one of them again in two. Spieksma *et al.* [13] examined the successive stages of the cascade impactor by scanning electron microscopy and detected no pollen fragments, but it is presumably difficult to observe, e.g. small size material from within pollen grains which reaches lower sampling stages.

In many previous studies only a limited number of days have been used to collect samples for immunochemical analyses. In our study the whole pollen period was monitored, because the activity peaks did not necessarily

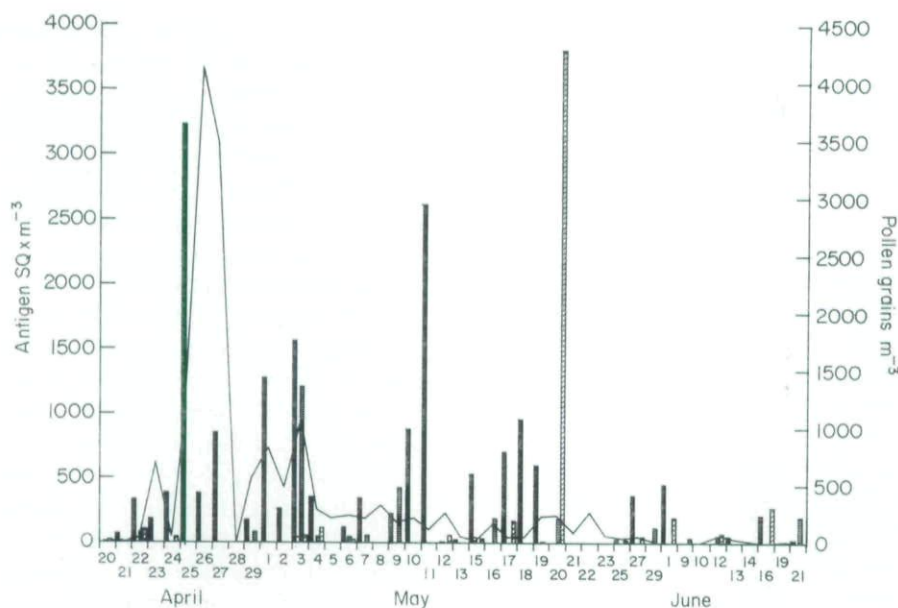


Fig 3. Antigenic activities in SQ units $\times m^{-3}$ in four (1–4) sampling stages of the SSBAS; Burkard counts (= B; pollen grains $\times m^{-3}$) from the same period, summer 1989. ■.1.□.2.▨.3.▤.4.—.B.

correlate with microscopical pollen counts. If sampled only during pollen peaks much of the allergologically important information may be lost. Thus, e.g. before the day of maximum pollen concentration, there was a peak in antigenic activity in filter stage 1 one day earlier (Fig. 3). This suggests that particles (over $7.2 \mu m$ in diameter) other than birch pollen were released from catkins before they opened totally to liberate pollen grains. It has been shown that antigens similar to pollen antigens are also found in other parts of the plants (in birch [19, 20]; in ragweed [21]; in oak [22]). The peaks of Burkard and SSBAS filter stage 1 were closely matched during the most intensive pollen period, but failed to coincide later in the season (Fig. 3).

Antigenic activities in filter stage 1 did not correlate with activities in any other sampling stage of the SSBAS (Table 1). Activities in filter stage 2 (for particle sizes between 7.2 and $2.4 \mu m$ in aerodynamic diameter) were lowest, indicating that birch pollen antigens are not often found in this size-class. More activity was found in filter stage 3 (particles below $2.4 \mu m$), indicating the existence of small micronic particles carrying birch protein. These two stages (2 and 3) were clearly significantly correlated (Table 1).

According to calibration experiments [16] all particles are trapped in filter stages 1–3 of SSBAS; only antigenic properties associated with atmospheric water vapour are detected in sample 4. Variations in antigenic activities in the condenser stage 4 were very different from all other stages (Fig. 3). Total activity was quite high, clearly

higher than in stages 2–3 together, indicating a high level of submicronic aerosol birch particles in the air.

Jensen *et al.* [15] showed a high correlation between Burkard results and antigenic activity in samples taken by high volume sampler. They did not fractionate the particles, but measured the total antigenic activity of birch and grass pollens in the samples collected with a high volume sampler. The particle size range collected by SSBAS stage 4 was not trapped in their study. Ratios of pollen concentrations and antigenic activities (IgG) were similar to ours.

During the first half of June there was a rainy period of about 1 week and the peak in stage 4 came after this (Fig. 3). The finding that rain resulted in an increase in airborne small antigenic fractions agrees with previous results about increasing concentrations of paucimicronic grass pollen allergen during days after rain [14]. Possibly, pollen sedimented on various surfaces releases its proteins when wetted. After drying, this material may again become airborne and is detected in samples.

This phenomenon could sporadically be more important for an allergic patient than the intact pollen grain count, since these molecular size birch particles are able to penetrate into the lower respiratory tract. These particles may result in the continuation of symptoms often reported after the peak pollen season [23].

Our results show that the ELISA method used is sufficiently sensitive for determining daily antigenic activities in air samples taken with low volume samplers. In this method binding of polyclonal rabbit anti-*Betula*

pollen IgG antibodies was measured. High specificity was not necessary in this case, because it has been shown that the immunodominant antigen is the main allergen of birch pollen [24,25]. The main allergen is thermoresistant and thus extraction temperature of 37°C could be used. Viander *et al.* [25] have also shown similar patterns in CRIE in binding of IgE antibodies before and after hyposensitization when using sera obtained from the same patients. The release of antigenic material from pollen grains starts quickly [26], about 70% of the total allergenic activity appearing in less than 30 min [25]. The amount of the thermostable antigen has been shown to increase further during 24 h extraction [25] and thus the extraction time used was not too long. Of course, also both enzymatic and physical degradation of the antigenic material occurs during incubation, and antigenic/allergenic activity has been detected in a fraction of small molecular weight substances which may be break-down products of the main allergen [27].

The particle dispersion has to be highly uniform [16] when such small samples as ours are used. (One 5 mm disc in our study represented 350–400 l of air). Analyses therefore have to be repeated at least four times. Operating the SSBAS is rather complicated and it is not suitable for instance for continuous trapping for pollen and spore information services, but only for special purposes.

Burkard tapes as such can also be analysed immunochemically by immunoblot microscopy [11]. Schumacher *et al.* were able to handle 2-h strips of Burkard tapes and found plenty of amorphous Bermuda grass antigen outside the pollen grains. The Burkard trap, however, is not properly calibrated and immunoblot microscopy is quantitative only by counting particles: for that purpose it is highly sensitive indeed. The high antigenic activities found in airborne particles smaller than intact pollen cast new light on the pollen allergy and aerobiological information service. In the testing of new drugs for pollen allergy, for instance, the results are compared with pollen counts. Comparison to airborne antigenic activity would be far more useful. An immunochemical analytic method would also give better support for information service. The SSBAS, together with the ELISA procedure, offers one such means for an improved information service.

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