

Visualization and quantification of birch-pollen allergens directly on air-sampling filters

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A new technique is presented to detect and quantify birch (*Betula pendula*)-pollen allergens directly on air-sampling filters. It is based on the use of specific antibodies, enzymatic reactions, and measurement by chemiluminescence or densitometry. The major pollen antigens are the most important birch allergens. The antibodies used recognize birch-pollen antigens which thus correspond to allergens. Calibration was done with a standardized extract of birch pollen for skin prick testing. The correlation coefficient for the logarithms of luminescence and the amount of birch-pollen allergen applied on filters was >0.98 in the range 0.04–200 SQ units. A similar correlation was found for the logarithms of the integrated densitometry values and the amount of birch-pollen allergen applied on filters. The number of major pollen-antigen particles or grains on filters could be estimated by counting the major stained spots produced by precipitated enzymatic products. The correlation coefficient was 0.90 for the logarithms of the number of counted major antigen spots and the calculated antigen amount obtained by luminometric measurements. Our results demonstrate that birch-pollen allergens can be determined directly on air-sampling, Teflon®-based filters by luminometry, optical density, or particle counting.

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Birch (*Betula pendula*) is an abundant tree species in the temperate zones. Its major pollen season lasts more than 4 weeks in the spring. Birch-pollen allergy may affect more than 20% of the population and causes various symptoms such as rhinitis and asthmatic reactions (1–3). Up to several hundred pollen grains per cubic meter of air are common in northern Europe during the pollen season (4). Birch-pollen grains contain proteins, which are sometimes released to form small particles with diameters of $<2\ \mu\text{m}$ (5, 6). The particles are found in the air also some weeks after the local pollen season and are often well distributed in the air (6). Particles with diameters of $<10\ \mu\text{m}$ are especially significant, because such particles can

penetrate much deeper into the airways than pollen (diameters of $>20\ \mu\text{m}$) and may cause lung symptoms. The major pollen-protein antigens are often the most important birch allergens (7). It is thus relevant to measure the concentration of airborne pollen antigens rather than that of the pollen grains for prevention of allergic reactions in man (5, 8).

Air sampling on a filter is commonly used to collect airborne substances in occupational hygiene. Polytetrafluoroethylene (PTFE), i.e., Teflon®-based, filters are frequently used, since they are water-resistant and have good collection properties and moderate pressure drop over the filter. After collection, the filters are currently extracted with a water solution, followed by the quantification of allergens in the extract (5, 6). However, we have found that birch-pollen-protein antigens stick to the filters and do not leach very much during the extraction procedure.

In this paper, we describe a technique for quantification of birch-pollen allergens directly on filters.

Abbreviations. AP: alkaline phosphatase; PTFE: polytetrafluoroethylene; TBS: aqueous solution of 0.15 M NaCl in 20 mM TRIS-HCl, pH 7.2; 0.2%-TBS: aqueous solution of 0.2% w/v Tween 20®, 0.5 M NaCl in Tris-HCl, pH 7.2.

The technique is based on binding of specific antibodies to the antigens and measurement of the enzymatic reaction products. Quantification of the antigens is done with either chemiluminescence or densitometry. The number of major stained spots corresponding to allergen particles can also be counted on filters.

Material and methods

Materials

Birch pollen and pollen counts were kindly supplied by the Palynological Laboratory, Swedish Museum of Natural History, Stockholm. The primary antibodies, rabbit IgG anti-*Betula* pollen antigens, were obtained from ALK (Hørsholm, Denmark), and the secondary antibodies, alkaline phosphatase-conjugated swine-IgG anti-rabbit IgG, from Dakopatts (Gentofte, Denmark). Water extracts of birch-pollen antigen for skin prick testing, Soluprick® SQ, standardized for skin prick testing in histamine equivalent prick (HEP), were bought from ALK. The extract contains 100 000 SQ units/ml, 10 000 biologic units/ml (9), and the amount of Bet v 1 is 23 µg/ml (information from ALK). Lumi-Phos® Plus was obtained from Lumigen, Inc. (Southfield, MI, USA), and BCIP/NBT from Bio-Rad (Hercules, CA, USA). Gel Bond® was obtained from FMC Bioproducts (Litex, Denmark), and filter paper from Munktell (Grycksbo, Sweden). ELISA plates (Black Cliniplate® for fluorometry) were from Labsystems OY (Helsinki, Finland). Polystyrene Petri dishes, diameter of 5 cm, were from Nunc (Roskilde, Denmark). A light-sensitive film Hyperfilm-ECL®, Amersham (Little Chalfont, UK), was used. All chemicals were of analytic grade. All filters used were made of PTFE with diameters of 47 mm and with a nominal pore diameter of 0.2 µm Sartorius (Göttingen, Germany). Filter holders made of translucent polycarbonate and with air inlet diameter of 6.5 mm were also from Sartorius. Air was suctioned through the filters with the Reciprotor vacuum pump, LECTROSTATIC (Skara, Sweden).

Instruments. The Luminoskan was from Labsystems OY (Helsinki, Finland), and the densitometer Elscript 400 from Hirschman (Munich, Germany).

Handling of PTFE filters

Three groups of filters were analyzed:

- 1) those for air sampling
- 2) those prepared by strewing pollen grains on filters (pollen-strewed filters)

- 3) those prepared with birch-pollen antigen solution (Soluprick SQ) to obtain the standard curve.

Air-sampling filters. The airflow rate through the filters was 10 l/min by stationary sampling on the roof of a building 11 m above ground level. Pollen was collected for 24 h on 18 days during the birch-pollen season in May 1996. After collection, the filters were carefully removed from their holders and kept in Petri dishes at -70°C until analysis.

Pollen-strewed filters. The filter was marked as described below and placed on the frit in an open glass filter holder, diameter of 47 mm (Millipore, Bedford, MA, USA). A few birch-pollen grains were spread from a small spatula resting on the rim of the filter holder, 10 cm above the filter collecting the grains, while air was suctioned through the filter at 7.5 l/min. The filter was left in the airflow for 15 min.

Adsorption and fixation of pollen allergens to PTFE filters. Air-sampling filters and pollen-strewed filters were placed carefully with the pollen-collection side up on the surface of isopropyl alcohol in a dish and left for 30 s. Then each filter was layered on the surface of 10 ml of a solution of 0.15 M NaCl in 20 mM TRIS-HCl, pH 7.2 (TBS), in a Petri dish and left overnight at 20°C.

Filters for the standard curve: marking the position of the spots. A frame was made of an aluminum plate (1×110×70 mm) by drilling holes through it matching the wells of an ELISA plate. A filter with a protecting paper piece on each side was laid on top of a glass plate, and the frame was placed over the protected filter. The filter was marked symmetrically on four sides to allow identification of the positions of the allergen spots. The marks were made with a sharp 5H graphite pencil through the holes of the frame on the protecting paper to make pressure marks on the filter. The marks corresponded to eight identifiable circular areas in the periphery of each filter.

Wetting the PTFE filter. The filter was placed carefully to float for 30 s on the surface of isopropyl alcohol. Then, it was laid on the surface of 100 ml of TBS in a shallow tray and left for 15 min.

Application of standard solutions. A box was humidified with a thick filter paper wetted with TBS placed on the bottom. The positions of the wells of an ELISA plate were marked as circles on a piece of ordinary overhead projection foil (diameter 6 cm). The foil was placed on top of the wet filter paper. A filter paper, 7 cm in diameter (Munktell,

Grycksbo, Sweden), was wetted in TBS and laid symmetrically on the plastic foil. The wet PTFE filter was laid on top of the filter paper so that the pencil marks coincided with the circles on the plastic surface visible through the filter paper. The standards, i.e., different dilutions of Soluprick SQ, were prepared and seeded as 20 μ l droplets on the PTFE filter in each circle. The droplets absorbed into the filter for at least 60 min. The filter was then placed with the samples facing up to float on the surface of 10 ml of TBS in a Petri dish and left overnight at 20°C.

Visualization and quantification of birch-pollen antigen on PTFE filters

Removal of unbound material. The filter was placed upside down on a fresh portion of 10 ml of TBS in a Petri dish and gently shaken for 60 min on a mechanical shaker. All subsequent incubations of filters were done in Petri dishes.

Blocking filters. The buffer in the dish was replaced by 10 ml of a solution of 0.2% w/v Tween 20, 0.5 M NaCl in Tris-HCl, pH 7.2 (0.2%-TBST), and shaken slowly for 60 min. Tween was used to avoid protein binding to the filter in the subsequent steps (10).

Incubation with primary and secondary antibodies. The blocking solution was replaced by 0.05% w/v Tween 20, 0.5 M NaCl in Tris-HCl, pH 7.2 (0.05%-TBST), and shaken for 15 min. It was replaced by 10 ml of a 1:1000 dilution of the primary antibodies in 0.05%-TBST and shaken for 60 min at 20°C. Three washing steps with 10 ml 0.05%-TBST and shaking for 15 min each preceded incubation for 60 min at 20°C in 5 ml of secondary antibodies (alkaline phosphatase-conjugated swine-IgG-anti-rabbit IgG) diluted 1:1000 in 0.05%-TBST. The filters were then washed three times in 10 ml 0.05%-TBST for 15 min, followed by a 15-min wash with 10 ml TBS, and kept in TBS, optionally overnight at 4°C.

Luminescence measurements. The filter was equilibrated in 10 ml of a solution of 0.1 M NaCl and 5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 9.5 (AP-buffer), by shaking for 15 min at 20°C. A rectangular 7×7 cm foil of Gel Bond® was placed with its hydrophilic side up on a level horizontal glass plate. A small water droplet between the foil and the glass was sufficient to hold the foil in place. On the hydrophilic foil surface was pipetted 100 μ l AP-buffer, and the filter was laid centered on top of the foil with its sample side up. Then 1 ml of Lumi-Phos Plus was spread with a pipette tip over the entire filter surface. After 3 min, a Gel Bond foil

(6×6 cm) was placed centered on top of the filter with its hydrophilic side against the filter. The set was covered with a thick absorbing paper (Type 1220, Munktell), and a roller was passed over to squeeze out excess reagent and air bubbles. The set was then carefully cleaned with soft absorbing paper and centered on top of a black ELISA plate. The set was placed so that the marks on the standard filters aligned with 16 wells of the ELISA plate, and was secured with plastic tape along its sides. A black film with transparent circular areas 7 mm in diameter precisely matching the wells was taped on the plate to allow selective luminescence in the spots. Sixty minutes after application of Lumi-Phos Plus, measurements were done with the Luminoskan® for 3.0 s at each area. Sixteen values were read for each whole filter (or eight values from a half filter).

Calibration was done with a standardized extract of birch pollen for skin prick testing (Soluprick SQ), and the amount of allergen measured in the present study is expressed in SQ units. The antibodies utilized recognize birch-pollen antigens, which represent the pollen allergens (information from ALK). In this report, antigens and allergens are used synonymously.

Luminography. The set was removed from the ELISA plate, and luminography was performed 120 min after addition of the luminescence reagent. In a darkroom, a sheet of Hyperfilm-ECL was laid on top of the assembly and left in a cassette for 60 s. Then, the film was removed and developed.

Alkaline phosphatase staining. The filter was removed from the assembly and washed twice with 10 ml water in a Petri dish on the mechanical shaker, each time for 15 min. The substrate BCIP-NBT was prepared by mixing 250 μ l of 5% BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 100% dimethylformamide with 500 μ l of 5% w/v NBT (nitro-blue tetrazolium chloride) in 70% dimethylformamide and 49.25 ml AP-buffer. The procedure was in principle as described by Altman (11). The filter was incubated for 60 min in 5 ml of the BCIP-NBT reagent, prepared immediately before use. The reaction was stopped by washing the filter twice with 10 ml water for 15 min at 20°C. The substrate BCIP/NBT was converted to dark-blue precipitates easily visible as dark spots on the filter where AP-enzyme was present.

Densitometric measurement of spots stained with BCIP/NBT on filter. The filter was scanned in the Elscript densitometer with a light filter for light absorbance reading at 436 nm and an optical slit 4×0.4 mm.

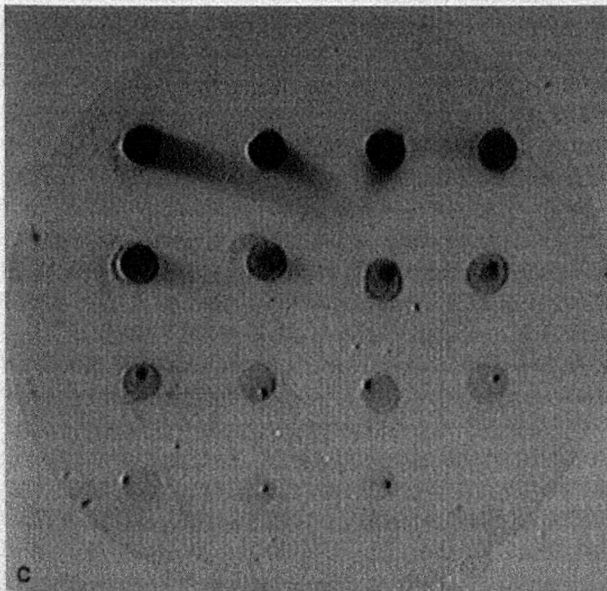
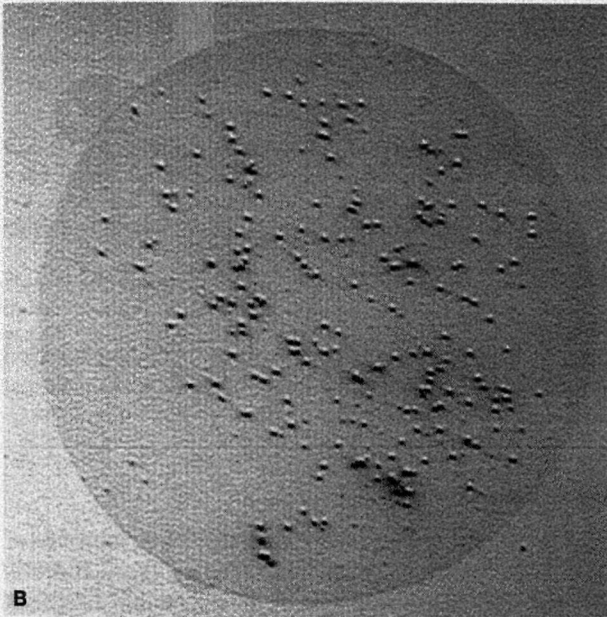
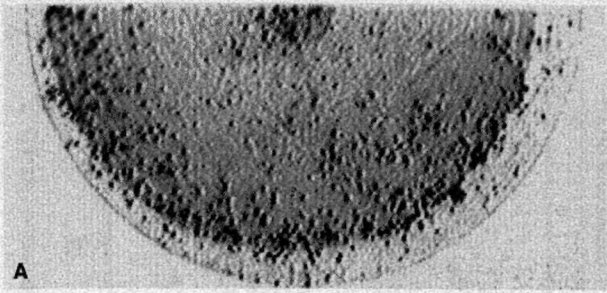


Fig. 1. Birch-pollen antigen on filters. Antigens were recognized with specific primary antibody (rabbit IgG anti-*Betula*-pollen protein) followed by alkaline phosphatase-conjugated secondary antibody (swine IgG anti-rabbit IgG) and stained with BCIP/NBT substrate. A) Half of air-sampling filter (7 m^3 air had been passed through whole filter); B) filter with strewn pollen grains; C) filter with 20- μl aliquots of serial dilutions of birch-pollen allergen, Soluprick SQ.

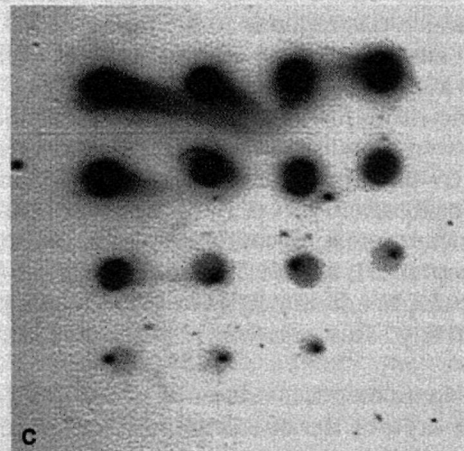
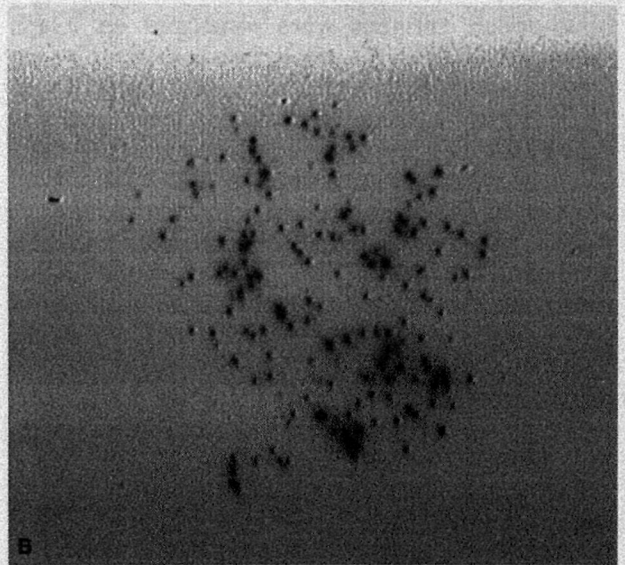
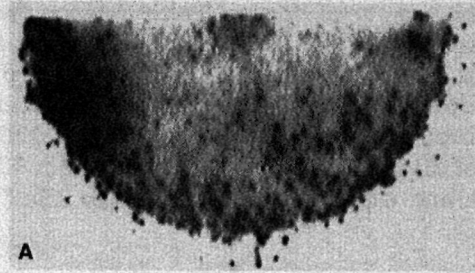


Fig. 2. Luminography of filters illustrated in Fig. 1. Alkaline phosphatase substrate used was Lumi-Phos Plus. Reaction proceeded for 2 h; then, photographic film was exposed for 60 s. A, B, and C correspond to those in legend of Fig. 1.

Counting the number of major stained spots. The number of major antigen particles on filters was estimated by counting the stained spots. The stained filter was photographed on a light box and also read with a black-and-white digitizing camera using 256 gray levels. The major particles were counted from the digitized pictures with the program UTHSCSA Image Tool for Windows, Version

1.27 (University of Texas Health Science Center, San Antonio, TX, USA) (public domain image analysis program, available on Internet: *FTP://maxrad6.uthscsa.edu*). Major particles were identified as those with a diameter of at least 0.35 mm (area of 10 pixels) with a gray level under 130 on a black-and-white scale between 0 and 255.

Results

Birch-pollen antigens were visualized on PTFE filters with BCIP/NBT, which was converted by the enzyme alkaline phosphatase to dark-blue spots. Fig. 1 shows stained air-sampling filters, pollen-strewed filters, and filters with dilutions of standard allergen solution. Air-sampling filters displayed particles of various sizes and a high proportion of minute ones barely visible to the naked eye. The strewed filters showed only major particles against a clear background. Our interpretation is that the major particles correspond to pollen grains, whereas the small ones correspond to protein particles collected in the air, which are not present in the pollen-strewed filters. The results obtained by luminography are illustrated in Fig. 2.

The concentration range generally used for the standard curve was 2–10 000 SQ units/ml. The lowest amount of allergen which gave a luminescence value above the background was 0.04 SQ units, which corresponds to 9.2 pg Bet v 1.

The luminescence measurements of different concentrations of the standard shown in Fig. 3 had a coefficient of linear regression of $r=0.98$. Similar results were obtained with optical density measurements ($r=0.97$) (graph not shown). For 13 luminescence calibration curves obtained over 3 months, with linear regression coefficients of >0.93 (criteria for acceptance), the coefficient of variations (CVs) for the slopes and intercepts were 15.8 and 13.2%, respectively. This variability was lower within a batch.

The luminescence values obtained from each circular area of the air-sampling filters were used to calculate the amount of antigen with the calibration curve. The total amount on each filter was calculated as four times the sum of the amounts in the measured areas, since this sum represents 25% of the total filter area. Fig. 4 shows the number of major particles counted on filters plotted against measured luminescence values. The correlation coefficient was $r=0.90$ for the filters prepared in the laboratory (strewed filters) and $r=0.77$ for the air-sampling filters. The good parallelism between the lines indicates the reliability of the procedures used. The results from the luminescence measurements of birch-pollen antigen in the air-sampling filters and the number of pollen grains per cubic

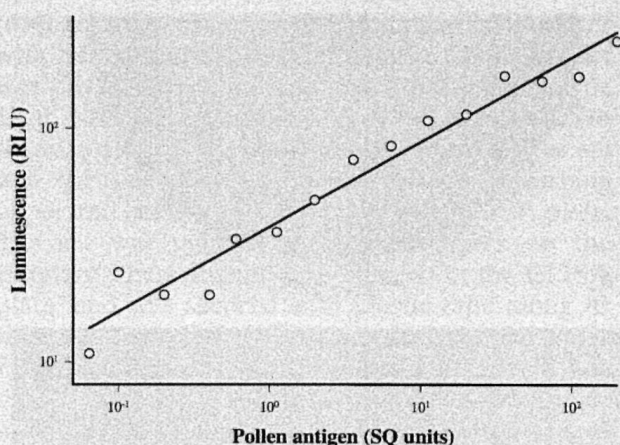


Fig. 3. Calibration curve. Luminescence readings (in relative light units [RLU]) plotted against amount of birch-pollen allergen, in 20- μ l samples from dilutions of Soluprick SQ expressed as SQ units. Linear regression coefficient was 0.98.

meter of air for the corresponding days, as reported by the Palynological Laboratory, Stockholm, are illustrated in Fig. 5 ($r=0.83$).

Discussion

White birch (*B. pendula*) releases large numbers of pollen grains into the air (12). Pollen from birch is one of the main allergenic sources in northern Europe. More than 20% of the population there suffer from IgE-mediated allergy mainly caused by water-soluble allergens from pollen grains. Common symptoms are conjunctivitis, lacrimation, rhinitis, and asthma (1–3). For several years, pollen grains have been sampled in the air, and the number of grains for each plant species counted per m^3 (pollen counts) in order to assess the risk of symptoms in allergic subjects and to offer forecast information (4). It has been shown that the allergens are proteins, which are released from pollen grains especially under moist conditions (5, 6, 8, 13). These proteins often form small airborne particles with aerodynamic diameters in the range 0.1–5 μ m (5, 6), which are found well distributed in the outdoor air not only at the time of the local pollen season but also weeks before and after the season (14). The protein particles thus occur widely distributed geographically and can maintain their allergenic properties for months (15). Therefore, ordinary pollen counts do not reflect the concentration in the air of particles with allergenic activity. Accordingly, it is more relevant to measure the concentration of airborne pollen allergens (5, 6, 8). This has not been done previously because earlier described methods for determination of their concentration in air needed improvement.

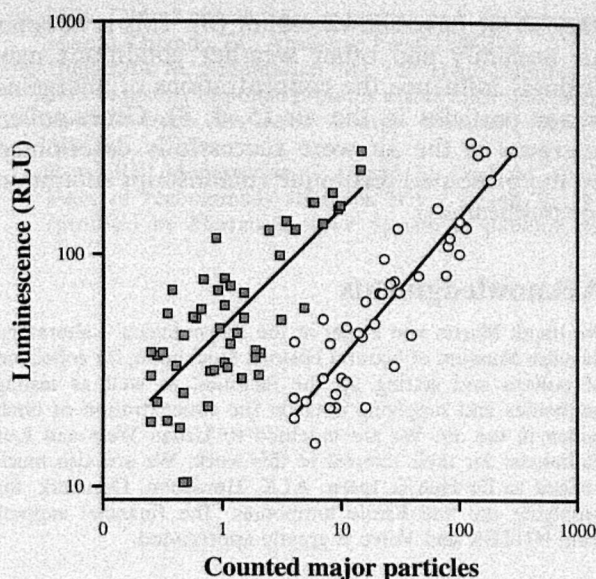


Fig. 4. Correlations between luminescence (in RLU) and stained major particles counted on filters. Circles represent results for pollen-strewed filters prepared in our laboratory ($r=0.90$). Squares are counts of particles on air-sampling filters (cf. Fig. 1A) ($r=0.77$). Major particles were identified in digitized pictures as spots with diameters of 0.35 mm or more and value lower than 130 on black-and-white scale of 0–255.

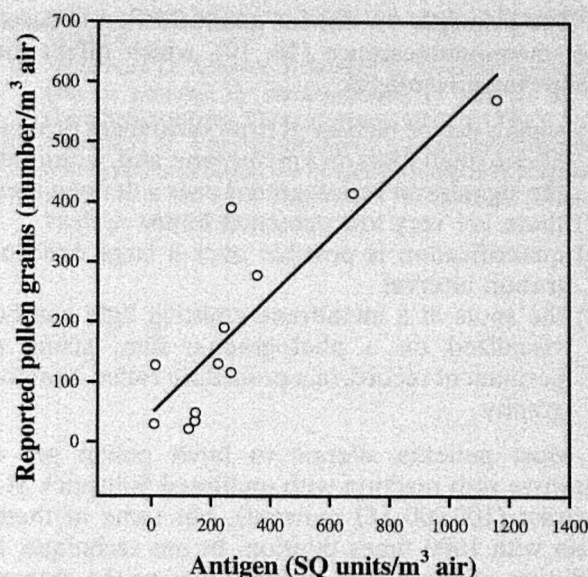


Fig. 5. Correlation for pollen concentration in air reported by Palynological Laboratory (Stockholm) and measured concentration of birch-pollen antigen per m^3 of air, obtained after passage of $7 m^3$ of air through filter on corresponding dates during birch-pollen season ($r=0.87$).

Upon inhalation, particles smaller than $5 \mu m$ in diameter are readily deposited in the lungs, whereas larger particles such as pollen grains do not penetrate so far into the airways. A significant fraction of the total allergens is inhaleable as small particles (5, 6) and may therefore be especially liable to provoke asthma. Furthermore, pollen grains from different trees and even different parts of one tree may have varying antigenic proportions (16). The importance and relative proportions of the allergens present in pollen grains and in particles are not easy to determine exactly, mainly for three reasons. Firstly, the proportions can be influenced by various meteorologic circumstances such as atmospheric humidity, wind strength, and wind direction (6). Secondly, the amount of allergenic activity can vary among pollen grains from a tree (15). Thirdly, the techniques for sampling and quantification presented up to now are not simple to use and have not yet been sufficiently developed. For instance, the sampler (SSBAS) used in some recent studies is expensive and not suitable for routine operation (5, 6). It is thus desirable to develop reliable and simple methods for collection of allergenic particles and pollen grains and for measurement of the total allergenic activity. Attempts in this direction have been presented recently (8).

It has been postulated that the immunodominant antigens are the main allergens of pollens (7). In

this study, we have used polyclonal antibodies binding to antigens from birch. The use of polyclonal antibodies recognizing several epitopes of allergens from a certain plant species may be proposed for the following reasons. Many allergenic proteins may be detected as opposed to the use of monoclonal ones, of which each one recognizes only one epitope. The use of only one epitope is a definite shortcoming in antigen antibody testing (17). Using one monoclonal antibody would miss a significant fraction of the total allergens present in many samples; e.g., birch pollen and its proteins. A test relying on only one epitope would also be susceptible to error because the antigen to be measured may be modified, and the epitope may become hidden by denaturation of the antigen or even be destroyed by oxidation before or after collection on a filter.

Measurements of allergenic activity have earlier been made by immunoassay (ELISA) of leaches into buffer solution from small disks punched from collection filters. Our results show that protein antigens originating from birch pollen stick to filters made of PTFE and do not leak during the analytic procedures. This is in accord with the general tendency of proteins to stick strongly to membranes used for blotting, such as PVDF, which is chemically related to PTFE. These facts allowed us to develop a technique using specific antibodies for quantification of birch-pollen protein antigens directly on collection filters.

The principle we use for quantification is based on chemiluminescence (18, 19), which offers the following advantages:

- 1) signals can be measured from substances present also in small spots on a membrane, and, if desired, the signals can be integrated over a defined area
- 2) there are very low detection limits
- 3) quantification is possible over a large concentration interval
- 4) the spots in a membrane emitting light can be visualized on a photographic film, giving a permanent record, in a procedure called luminography.

Most patients allergic to birch pollen get a positive skin reaction with undiluted Soluprick SQ extract (100 000 SQ units/ml), but some of them also with 1000 times dilution. In our technique, a dilution of more than 50 000 times of the extract can give measurable luminescence. The most diluted standard dot giving a luminescence reading above the background contained 0.04 SQ units, corresponding to 9.2 pg Bet v 1. The technique thus permits detection and quantification of picogram amounts of birch-pollen allergen in the air. The quantification of allergen in filters can be made over a wide concentration range of over three orders of magnitude.

For the plot of luminescence against the number of pollen major particles found on filters, the correlation coefficient was $r=0.90$ for the filters prepared in the laboratory and $r=0.77$ for the air-sampling filters, as illustrated in Fig. 4. The larger particles collected on filters correspond to pollen grains, whereas the smaller ones are antigenic particles, which cannot to be seen in the pollen-strewn filters (cf. Fig. 1A and B). This is in accord with the observation that the air-sampling filters contain, in addition to pollen grains, minor protein particles which increase the total luminescence. The two types of pollen grains studied gave results located in two separate clusters. The parallelism between the lines indicates the reliability of our quantification technique.

It is interesting to find a correlation ($r=0.83$, cf. Fig. 5) between the luminescence measurements of birch-pollen-protein antigen in the air-sampling filters and the number of pollen grains per cubic meter in air, as reported by the Palynological Laboratory, Stockholm, although the sampling locations were 4 km apart, and different procedures were used at collection as well as counting. A correlation can be expected, since the protein particles originating from pollen grains should be more abundant the higher the concentration of pollen grains in the air. However, for single days and even within hours of a day, the relation may

vary, as we have shown earlier (8). This is because air humidity and other weather conditions may strongly influence the concentrations of allergenic minor particles in the air (5, 6, 8). Grass-pollen allergens in the air were successfully determined by the presented technique (manuscript submitted for publication).

Acknowledgments

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