

Comparison of direct immunostaining and electroimmunoassay for analysis of airborne grass-pollen antigens

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Sensitive pollen-allergic patients have been reported to show allergic symptoms not only during the pollen release of allergenic plants but also both before and after the pollen season. Symptoms before the season are evidently provoked by small-sized particles originating partly from developing pollen grains, partly from other plant parts. After the pollen season, antigenic material settles on various surfaces, which thus form a new source of allergenic material. Measuring the allergen concentrations in indoor and outdoor environments demands an effective sampling method and a rapid and sensitive immunochemical analysis, especially for particles of small-sized fractions which are not detected in microscopic analyses. The efficiency of an ELISA and an immunochemical staining method was tested with monoclonal IgG against Phl p 5, the main grass allergen. The Burkard trap and MPC impactor (Marple personal cascade impactor with six-stage particle size fractionation) were compared. The sampling was carried out in southwestern Finland in the summer of 1994. The number of grass-pollen antigen spots greatly exceeded the simultaneous pollen count, indicating considerable antigen activity outside the pollen grains. The counts were especially high in small-sized fractions after the pollen season, when hardly any airborne pollen was found. Spots and pollen divided according to size were highly intercorrelated, indicating that the threshold values used were appropriate.

Birch (*Betula* spp.) and grass (Poaceae) pollens are the most important cause of pollinosis in Scandinavia. Sensitive pollen-allergic patients have been reported to show allergic symptoms not only during the pollen release of allergenic plants but also both before and after the pollen period. Symptoms appearing before the season are evidently provoked by small-sized particles (1), sometimes by nonpollen allergens but usually by exactly the same proteins which are also the main pollen allergens. These particles originate partly from developing pollen grains (2), partly from other plant parts (3, 4).

The methodological keystones to demonstrating allergenic activity in small-sized particles are 1) sampling in different fractions according to the aerodynamic size of the particles in both outdoor and indoor situations, and 2) finding a method of

immunochemical analysis sufficiently sensitive to low concentrations of antigenic activity.

Particles in small-sized fractions can penetrate deep into the respiratory tract. Grass-pollen antigens (Lol p 9=Lol p 5; recommendation of International Union of Immunological Societies [IUIS] subcommittee for allergen nomenclature) are known to occur in starch granules (5–7), which are of micronic sizes, ranging from 0.6 to 2.5 µm; they are respirable, and hence may trigger an asthma attack (6, 8, 9). Another group of small particles which become airborne from pollen before the season is that of Übisch bodies, i.e., orbicules (2, 10). These outgrowths of the intine are common in various plant species (10, 11). Birch-pollen antigens are found in the orbicules, ranging in size from 2 to 4 µm (10), and, according to Nakamura et al. (12), also in amyloplasts.

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The ELISA (enzyme-linked immunosorbent assay) was developed to analyze the antigenic activity of a particle sample in liquid. Takahashi et al. (13) introduced a direct immunostaining method of counting the number of antigenic particles of different sizes in a Burkard sample.

In the present study, we compared these two immunochemical methods, using particle samples taken from the air by samplers with and without particle size fractionation. The aim was to find a method of analysis which would also provide information on sizes of allergenic particles in an air sample.

Material and methods

Sampling procedures

The grass-pollen antigen samples were collected during the pollen season in the summer of 1994. Pollen sampling was performed 1) with a Burkard 7-day recording spore and pollen trap (Burkard Manufacturing, Rickmansworth, UK [14]), and 2) with an MPC impactor (Marple personal cascade impactor; Andersen Manufacturing [15]).

The Burkard trap, with a suction volume of 9 l/min, has no particle size fractionation. The trap was placed on the ground adjacent to a hayfield in a rural area (12 km from the city of Turku). The Melinex tape was coated with vaseline-glycerol adhesive and was replaced weekly.

With a suction volume of 2 l min^{-1} , the MPC impactor was situated 2 m from the Burkard trap, and the filters were changed every 7 days to obtain samples sufficiently loaded with antigen. This length of sampling time was made possible by the availability of current (this sampler usually runs off a battery supply). The impactor has six-stage particle size fractionation (the cutoff points are 9.8, 6.0, 3.5, 0.93, 0.52, and $<0.52\text{ }\mu\text{m}$), and the sampler thus reliably collects all micronic size particles with antigenic activity. The Mylar filters were coated with the same vaseline-glycerol adhesive as the Burkard tapes.

All samples for immunochemical analyses were stored at 4°C .

Analysis of airborne pollen sample

Microscopic analyses. After 7 days, sampling was completed. The Burkard tapes were split in half longitudinally (parallel to the time-axis) and then cut into 24-h segments corresponding to each sampling day. The microscopic analyses were carried out with $\times 400$ magnification. All individual grass-pollen grains were counted from one of the halves of the Melinex tapes, on a 2-h basis. Grass pollen was divided into three groups for counting:

small- ($<30\text{ }\mu\text{m}$), medium- ($30\text{--}50\text{ }\mu\text{m}$), and large-sized grains ($>50\text{ }\mu\text{m}$ in diameter). The cutoff values were based on the known sizes of the grass pollen occurring generally in the vicinity of the research area: *Alopecurus pratensis* ($25\text{--}34\text{ }\mu\text{m}$), *Phleum pratense* ($32\text{--}40\text{ }\mu\text{m}$), *Dactylis glomerata* ($30\text{--}38\text{ }\mu\text{m}$), *Poa* spp. ($25\text{--}32\text{ }\mu\text{m}$), and *Secale cereale* ($47\text{--}65\text{ }\mu\text{m}$) (16, 17).

Immunostaining. The Mylar filters of the MPC impactor were cut in two. The other halves of both the Mylar filters and the Burkard tapes were placed in contact for 1 h with nitrocellulose membranes (10×55 , $36\times 24\text{ mm}$; Bio-Rad Laboratory, CA, USA), which were treated by direct immunostaining (13). The immunochemical analysis was carried out with monoclonal IgG antibody against Phl p 5, produced in mouse (kindly provided by Dr Carsten Schou, ALK, Denmark). Then, the samples were incubated in AP (alkaline phosphatase)-conjugated rabbit antibodies to mouse immunoglobulins (DAKO A/S). After treatment with BCIP/NBT (5-bromo-4-chloro-indodyl phosphate/nitroblue tetrazolium, a phosphatase substrate kit [Kirkegaard & Perry Laboratory, Gaithersburg, MD, USA]), the grass-pollen allergenic particles appeared as purple spots on the nitrocellulose membrane.

ELISA (enzyme-linked immunosorbent assay). ELISA was carried out only for the Mylar filters of the MPC samples. Half of the Mylar filters from the six stages combined were shaken for 1 h in 5 ml of PBS (phosphate-buffered solution, pH 7.4) in a sterile tube. The lyophilized samples were analyzed by IgG ELISA (18) using monoclonal antibodies against Phl p 5 and rabbit anti-IgG antibody to mouse immunoglobulins conjugated with HRP (horseradish peroxidase, DAKO A/S). The results are given in SQ units (standard quality, ALK, Copenhagen).

Image analyses and statistical analysis. The number and size of the purple spots on the nitrocellulose membranes (Burkard samples) were determined automatically from manually selected 2-h segments with an image analysis system (MCID, Model M4, Imaging Research, Inc., Canada). The device consists of 1) an illuminator (Northern Light, Model B 90, Imaging Research, Inc., Canada), 2) a macro-zoom lens ($18\text{--}108\text{ mm}$), 3) a CCD camera (Hamatsu c 3077), and 4) the image analysis system (MCID, Model M4, Imaging Research, Inc., Canada). The image of the tape was adjusted to fill the screen; consecutive segments, each representing a 2-h sampling, were manually selected, and the number of spots in each segment was automatically counted. The system can resolve particles larger than $10\text{ }\mu\text{m}$.

The spots counted were divided into three groups by size for the statistical analyses. The spots are reported to be about three times larger than the whole particles (19). Therefore, arbitrary spot sizes were chosen as follows: small ($<100\text{ }\mu\text{m}$), medium ($100\text{--}300\text{ }\mu\text{m}$), and large ($>300\text{ }\mu\text{m}$ in diameter).

The Pearson correlation coefficient was calculated with the SYSTAT program library (20). The number of spots was compared with the total pollen count from the other half of the Melinex tapes from the Burkard samples. The MPC samples were also screened with a microscope to detect occasional intact pollen grains.

Results

In southwestern Finland, the summer of 1994 was dry and sunny. The grass pollen season began at the end of June and reached an unusually high peak pollen period in the Turku area (21).

The numbers of small-, medium-, and large-sized pollen grains and spots during the study period, analyzed from the Melinex tapes, are shown in Fig. 1. Small spots were most frequent throughout the season. Both large pollen grains (i.e., cereal pollen) and large spots were very rare. The sporadic spots (and pollen) occurring in the MPC filters were too few to be presented as a diagram.

The daily count of grass pollen was regularly lower than the number of spots in the small- and medium-sized categories. Small-sized spots were more common than medium-sized ones; for pollen,

the situation was the reverse. The results for 16 July were an exception, with an enormous number of medium-sized pollen compared to the number of spots. The microscopic analyses showed many of the pollen grains to be empty of cytoplasm. At the end of the pollen season, there were many small- and medium-sized spots in comparison to the low number of grass-pollen grains (Figs. 1 and 2).

At the end of the study period, spots were present in high numbers while the air was almost empty of grass-pollen grains. No large pollen grains or big spots were observed.

The numbers of small- and medium-sized antigen spots and pollen were intercorrelated (Table 1). The number of large pollen grains was correlated highly significantly only with the number of large spots.

Discussion

The main objective of this study was to determine the concentrations of small-sized grass antigenic particles carried from a large hayfield into the environment, and the relationship between this count and the grass-pollen count. The MPC sampler was selected for the tests because it is theoretically ideal for collecting micronic particles (15). Sampling with the Burkard trap is the routine method for airborne pollen counts. The Burkard trap is not efficient at catching small particle sizes (below $4\text{ }\mu\text{m}$ in diameter) (22). However, the Burkard trap has been used previously to analyze various particle sizes in air samples (13, 19,

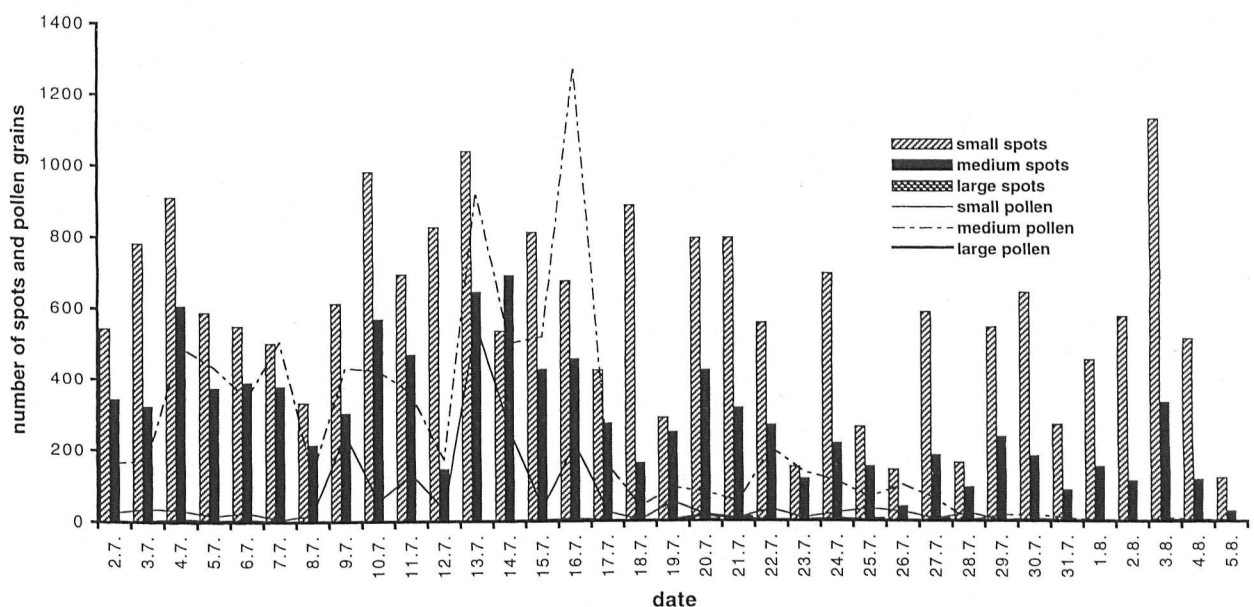


Fig. 1. Mean daily number of small-, medium-, and large-sized spots (<100 , $100\text{--}300$, $>300\text{ }\mu\text{m}$) and pollen (<30 , $30\text{--}50$, $>50\text{ }\mu\text{m}$ in diameter, respectively) during study period (2 July–8 August 1994). Counts of airborne pollen were obtained from continuously operating Burkard trap ($n=35$ days).

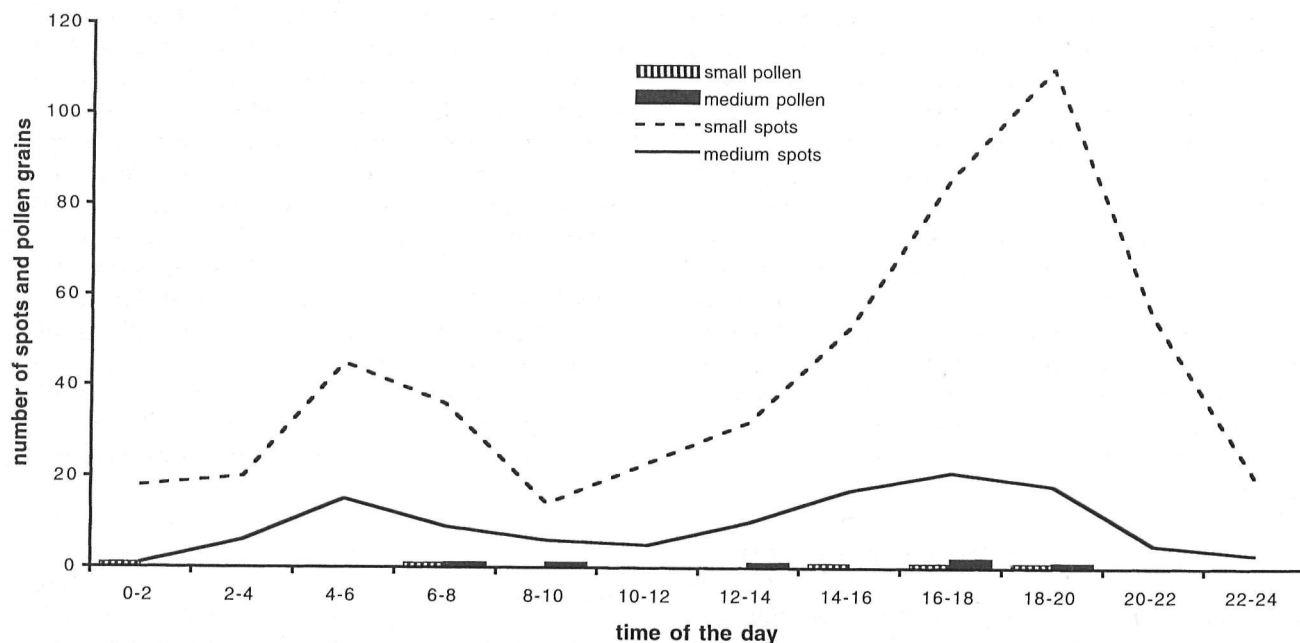


Fig. 2. Average number of small- and medium-sized spots and pollen grains during last 8 days on 2-h basis. There were no large spots and pollen.

23), so that some material for comparisons already existed.

Immunochemical electroimmunoassay (EIA) and radioimmunoassay analyses have been widely used to measure antigen concentrations in biologic materials. The results may be given in ng g^{-1} of dust or in SQ units (standard quality, ALK, Copenhagen, Denmark), an arbitrary unit which cannot be directly applied to the quantities of the present sample. These methods are generally time-consuming and thus not very suitable for the daily routine analysis of airborne antigen concentrations, as, for instance, for public information services. Thus, a rapid, direct (immuno)staining method of sampling and automatic image analysis could be a solution. Two methods, ELISA and immuno-staining, were compared for accuracy and conveni-

ence, focusing particularly on the analysis of micronic particles.

EIA is convenient when the sample size of small particles is large enough; for instance, when the sampler is sufficiently effective to yield a sample with antigenic activity high enough for chemical analyses. Often relatively long sampling times are needed to capture a sufficient number of particles, so that short-term changes in antigenic concentrations become impossible to observe.

Immunostaining with automatic image analysis is a fairly quick method. It gives the count of allergenic particles in a sample but it does not provide adequate evidence of either particle size fractioning or antigenic concentrations. For very small particles, this might be only a minor dis-

Table 1. Correlation coefficients between small, medium, and large spots (<100, 100–300, and >300 μm in diameter, respectively), and small, medium, and large pollen grains (<30, 30–50, and >50 μm in diameter, respectively) in 1994

	Spot				Pollen grain		
	Small	Medium	Large	Total	Small	Medium	Large
Spot medium	0.695***						
Spot large	0.069	0.088°					
Pollen grain small	0.421***	0.478***	0.028				
Pollen grain medium	0.471***	0.511***	0.050				
Pollen grain large	0.052	0.063	0.923***				
Spot total					0.648***	–0.008	
Pollen grain total	0.494***	0.545***	0.146**	0.554***	0.475***	0.526***	–0.011

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ° $P = 0.1$.

advantage, since the count may be more important than the entire volume. Irrelevant counts of empty pollen are not needed. An important advantage of the immunostaining method is that it allows detection also of nonpollen particles, including at least the largest antigen particles, and that it therefore gives more detailed information on air quality than the amount of pollen alone. According to Takahashi et al. (19), the spots vary in size, being conceivably 2–3 times larger than the corresponding pollen grain or other antigenic particle, while pollen of equal size can yield different sized spots (24). The method leads to misinformation concerning particle size when the particles are aggregated or partly overlapping, or when there is variation in color intensity within the spots. Sometimes one spot may be counted as a larger number of small ones, depending on the threshold value selected. This occurs especially at the edges of the spots, and the numbers of small spots may therefore be overestimated in the analyses of Burkard samples.

The adjustment of the threshold value is problematic when the number and intensity of spots varies greatly in samples from the same observation period. This problem could be alleviated if a sampler with reliable particle size fractionation were available, and only spots within a certain size range were counted in the sample.

Unfortunately, counts of the spots of the MPC samples analyzed from the nitrocellulose membrane could not be carried out because the spots on the MPC filters were very rare and sporadic, indicating the absence of really small particle sizes (such as starch granules or orbicules) in the air in the vicinity of the hayfield. Knox et al. (6) have reported high starch granula counts after rainy periods. Our sampling period was almost totally rainless, so that the MPC results do not tell us anything about the situation in rainy conditions. The MPC impactor failed to trap the pollen size fraction effectively; all the spots accumulated near the airflow hole in every stage and were therefore indistinguishable. However, we were able to demonstrate that “flying orbicules” are not a common phenomenon in southwestern Finland. The antigen concentrations were too low for ELISA analyses of the MPC samples.

Since the weather of the study period was dry and sunny, it seems evident that antigenic activity also remained high in settled dust over the whole period (data not shown here). Some of the antigenic activity in the samples may thus have been caused by refloats of pollen grains. The smallest particles detected in the Burkard samples may be the smallest intact pollen grains or clumps of cytoplasm and other plant parts, but there were hardly any particles below 4 µm in aerodynamic size.

The size of many of the spots was larger than that of most small grass-pollen grains. The color intensity of the spots varied.

It seems evident that empty pollen – for example, on 16 July – had released antigens into the air, and that the particles could be identified in the sample as small spots days later. Worth noticing also was the presence of small-sized spots in high numbers at the end of the study period. Small-sized particles can stay much longer in air than whole, heavy pollen grains. The small number of large pollen and spots was due to the great distance from the wind-pollinated rye fields and to the general circumstances of diffusion.

According to Valenta et al. (25), group 5 allergens cause symptoms in 80% of all patients sensitive to grass pollen. The monoclonal antibody against Phl p 5 used in this study should have stained a large amount of the grass-pollen antigens of the samples, since antigenic and allergenic cross-reactivity has been shown between grass-pollen allergens from groups 5 and 9 (26–28).

In conclusion, we are not yet able to introduce a rapid immunochemical method for routine pollen-antigen analysis. The immunostaining method still needs further modification, while automatic image analysis also has certain disadvantages. The air-intake volume of the MPC sampler was not sufficiently high; another sampler type is needed, with higher suction volume and particle size selection (29). With increasingly reliable size fraction of the sampler, image analysis will be easier to carry out also for small particle sizes, which, especially before the pollen season, sometimes occur in high concentrations (1) and provoke pre-season symptoms. These particles are never detected in the visual analysis of Burkard samples but must be detected immunochemically.

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