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Prolonged antigenic activity of birch and grass pollen in experimental conditions

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Pollen of birch (*Betula*) and grass (Poaceae) are the main cause of seasonal allergy in northern European countries. Allergic symptoms may also occur outside the local pollen season. From the point of view of allergic sufferers, it is important to know how long pollen retains its sensitising power. The maintenance of antigenic activity under different experimental conditions was studied over a period of one year. Pollen of birch (*Betula pendula* Roth., *B. pubescens* Ehrh.) and timothy (*Phleum pratense* L.) grass was collected in the summer of 1995 in south-western Finland. Pollen samples were incubated in petri dishes in separate desiccators, at 20°C at relative humidities of 23%, 33%, 43%, and 54%, which coincides with typical values in Finnish homes. Control pollen samples were stored under room conditions. The samples were analysed with IgG-ELISA (enzyme-linked immunosorbent assay) using monoclonal (specific to Bet v 1) and polyclonal birch pollen antibodies and Phl p 5 antibody, with SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and IgE-immunoblotting using sera from birch pollen allergic subjects. Birch and grass pollen antigenic activity remained high in all treatments during the entire study period. Different relative humidities had no detectable effect on the antigen concentrations.

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Pollen of birch (*Betula*) and grass (Poaceae) are the main cause of seasonal allergies in northern Europe. Pollen grains are generally considered to be a risk factor for pollen-allergic people during the pollen season, but there is clinical confirmation that allergic symptoms may also occur outside the local pollen season (Wallin et al. 1991). These off-season symptoms may be caused by long-distance transported pollen (Hjelmroos 1991), pollen in settled dust (Yli-Panula & Rantio-Lehtimäki 1995), airborne small size pollen particles (Ekebom et al. 1996, Pehkonen & Rantio-Lehtimäki 1994) or allergens of cross-reactive species (Pehkonen & Rantio-Lehtimäki 1995) or by non-pollen material originating from allergenic plants (Fountain et al. 1992, Grote & Fromme 1986).

Birch pollen maintains its antigenic activity in outdoor settled dust for several weeks after the pollen season (Yli-Panula & Rantio-Lehtimäki 1994). In indoor settled dust, Bet v 1, the major birch pollen allergen (Yli-Panula & Rantio-Lehtimäki 1995) and Phl p 5, a major grass (*Phleum pratense* L.) pollen allergen (Yli-Panula 1997) have been determined as late as five weeks after the end of the pollen period. In Norwegian schools, antigenic activities of birch and timothy pollen were observed in dust samples collected as late as March of the following year (Dotterud et al. 1997).

Allergologically very important allergens in Scandinavia are the Bet v 1 allergen and the Phl p 5 allergen which shows high homology with the group 5 allergens of other common grass species (Ong et al. 1996) and thus can be used to represent the grass pollen family in most cases. Bet v 1 is probably involved in pathogen resistance within the pollen grain (Breiteneder et al. 1989, Swoboda et al. 1995b); this

property would be needed especially when the pollen grain leaves the anther and during fertilisation. The meaning of the synthetization of Phl p 5 allergens is not known.

Earlier findings of pollen antigenic activity in indoor settled dust after the pollen period (Dotterud et al. 1997, Dybendal et al. 1989, 1991; Yli-Panula & Rantio-Lehtimäki 1995, Yli-Panula 1997), and the assumptions that air temperature and relative humidity may affect the antigen concentration of small size particles in outdoor air (Pehkonen & Rantio-Lehtimäki 1994), give rise to the following questions: a) How long is it possible for birch and grass pollen to maintain antigenic activity? b) What are the effects of different relative humidities on the antigenic activities of birch and grass pollen? c) How do Bet v 1, Phl p 5 allergen and birch antigen concentrations vary over an extended period?

MATERIALS AND METHODS

Sampling of birch and grass pollen and laboratory experiments

Catkins of randomly chosen birch trees (*Betula pendula* Roth., *B. pubescens* Ehrh.) were collected in paperbags on the first day of the birch pollen season in May 1995 (Finnish Pollen Bulletin 1995). Spikes of timothy were collected during one week at the beginning of the grass pollen season. The birch pollen was separated from the catkins using a sieve with 140 µm pore size; the grass pollen was sifted with a 297 µm pore size.

The birch and grass pollen grains were stored in a freezer at –70°C for a few days until the start of the experiment to avoid mold and bacterial growth. Birch and timothy pollen (about 6 g each) were placed in open petri dishes in separate desiccators. The following relative humidities (RH) were adjusted with binary satur-

ated aqueous solutions: 23% (potassium acetate, $\text{KC}_2\text{H}_3\text{O}_2$), 33% (magnesium chloride, MgCl_2), 43% (potassium carbonate, K_2CO_3), 54% (magnesium nitrate, $\text{Mg}(\text{NO}_3)_2$) at 20°C (Greenspan 1977) and day and night (7 hours) light alteration. The recommended RH in Finnish homes is 30–40%, but this level is seldom achieved in winter because of central heating. Control samples of pollen were kept at room conditions (RH varied 17–29% and temperature 19–23°C).

Immunochemical analyses

IgG-ELISA (enzyme-linked-immunosorbent assay) analyses. – For each analysis, 2 mg of pollen was weighed from each treatment. The intervening time before the taking of the samples varied from 2 weeks to two months (Figs. 1, 2 & 3) during one-year study period. The samples were analysed by a modification of the IgG-ELISA method developed for outdoor air analyses of birch pollen antigens (Rantio-Lehtimäki et al. 1994). The analyses was carried out like in our earlier study (for details see: Yli-Panula & Rantio-Lehtimäki 1995) except for the following alterations. The weighed pollen samples were incubated in 2 ml of phosphate-buffered saline (PBS), pH 7.4, shaken 1 hour at 4°C, and centrifuged at 14 000 rpm for 10 min. The dilution series were from 1:1000 to 1:10⁶ in PBS. Birch and Bet v 1 antigen was detected by means of polyclonal and monoclonal antibodies against *Betula* pollen produced in rabbit ALK/AS, Copenhagen, Denmark). Grass pollen allergen was detected with murine monoclonal antibody against Phl p 5 (kindly provided by Dr Carsten Schou, ALK, Denmark) diluted 1:1000 in PBS-BSA (bovine serum albumin 0.5%) using HRP-conjugated rabbit antibodies against the mouse IgG (DAKO A/S, 125 µl/well of 1:1000 dilution in PBS-BSA-Tw (Tween 0.5%)).

The *Betula verrucosa* pollen extract [100 000 SQ (standard quality unit) ml⁻¹ – dilutions of 1:100, 1:500, 1:1000, 1:5000 and 1:10 000] and *Phleum pratense* pollen extract (100 000 SQ) used as standards (dilutions of 1:100, 1:500, 1:750, 1:1000 and 1:5000) were provided by ALK, Copenhagen. About 1000 SQ-units corresponds to 23 ng antigens per ml (ALK, Copenhagen).

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and IgE. – For immunoblotting, sera from thirty birch pollen allergic subjects and five non-allergic individuals were tested for IgE-antibodies against birch pollen antigens using the *Betula verrucosa* pollen extract (ALK, Copenhagen). The serum pool for the analyses was prepared from equal volumes of 10 sera of allergic subjects based on the results of immunoblotting. Three sera were chosen for the negative control sera pool.

20 mg of birch pollen from each treatment was weighed every other week (Fig. 4). SDS- and IgE-immunoblotting were carried out from birch pollen samples of the first 18 weeks (16 weeks at room conditions). All samples were extracted in 0.125 M NaHCO_3 (pH 7.0, 1:10 w/v) with 0.015 M sodium azide and shaken for 24 h. The amount of each extract was directly proportional to the raw material. Proteins from the supernatants and molecular weight markers (Rainbow, Amersham, England) were separated using SDS-PAGE (Laemmli 1970) in a 5–17.5% gradient gel under non-reducing conditions. Following Towbin et al. (1979), separated proteins were transferred to nitro-cellulose sheets with pore size 0.2 µm (Schleicher & Schuell, Germany) using Transphor device (LKB, Sweden) for IgE-immunoblotting.

Allergens were determined according to Bengtson et al. (1985). Contrary to the standard procedure, radioactively labelled allergens were visualised by a sensitive phosphor imager system rather than by X-ray films (Bio Rad laboratories, USA). To confirm the comparability of different exposures, ¹²⁵I-conjugated anti-IgE antibodies reference discs were used (Aukrust & Aas 1977). Bands were visualised and band intensities were measured by Phosphor Analysis 1.1 software (Bio Rad laboratories, USA). The results are presented as instrument counts (IC; see: Molecular analyst® 1992).

Statistical analyses

Spearman's correlation coefficients were calculated for the non-normally distributed data of IgG-ELISA and IgE-immunoblotting by CORR procedure (SAS 1993).

The curves for the data of the IgE-immunoblotting analyses in Fig. 4 were fitted according to the distance-weighted least squares smoothing procedure in Axum 4.0 (Trimetric Inc. 1994).

RESULTS

Antigenic activity of birch and grass pollen was found throughout the one-year study period. Birch-pollen antigenic activity (Figs. 1, 2) was higher than that of grass pollen (Fig. 3). Birch antigens were detected in all samples with both the ELISA method and IgE-immunoblotting. Distinguishable high antigen concentrations appeared randomly in the ELISA samples.

Bet v 1 antibody proved to be more efficient in binding allergens than the polyclonal antibody, due to the higher specificity of the former.

The ICs of the major birch pollen allergen (Fig. 4) were highest at the beginning of the study period (880 000 IC in room conditions). Figure 4 clearly shows the variation in allergens: a rapid decrease to the lowest level in four to six weeks, relative stabilisation at this level and an increase in

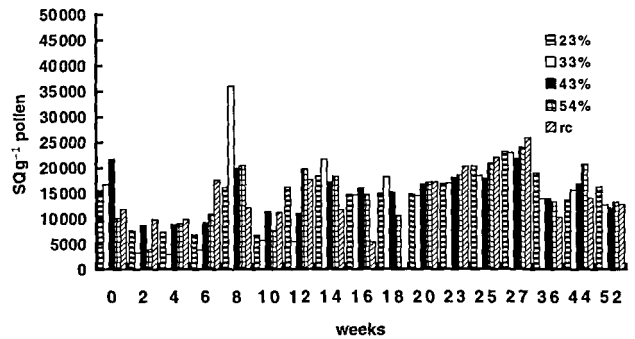


Fig. 1. Birch-pollen antigenic activity in SQ units g⁻¹ of pollen grains. Pollen samples were incubated at 20°C at the relative humidities 23%, 33%, 43% and 54%, respectively and in room conditions (rc). Number of measurements: 17 (16 in rc) during 52 weeks study period.

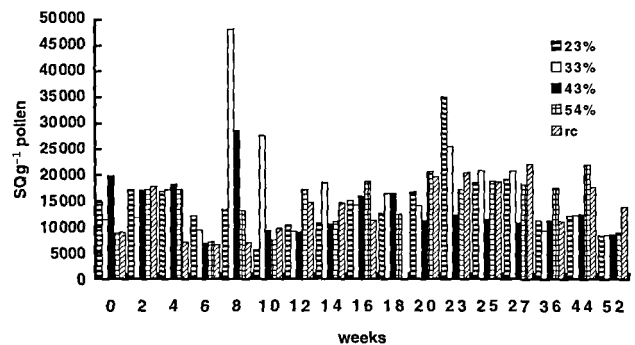


Fig. 2. Bet v 1 allergenic activity (SQ units g⁻¹ of pollen grains). Pollen samples were incubated at 20°C at the relative humidities 23%, 33%, 43% and 54%, respectively and in room conditions (rc). Number of measurements: 17 (16 in rc) during 52 weeks study period.

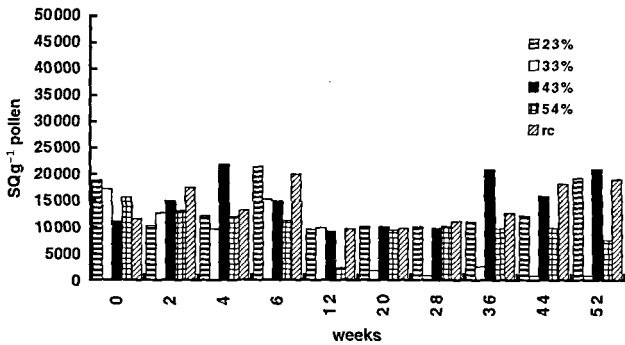


Fig. 3. Phl p 5 allergenic activity (SQ units g⁻¹ of pollen grains). Pollen samples were incubated at 20°C at the relative humidities 23%, 33%, 43% and 54%, respectively and in room conditions (rc). Number of measurements: 10 (9 in rc) during 52 weeks study period. (The sample stored at the humidity of 33% had mold growth).

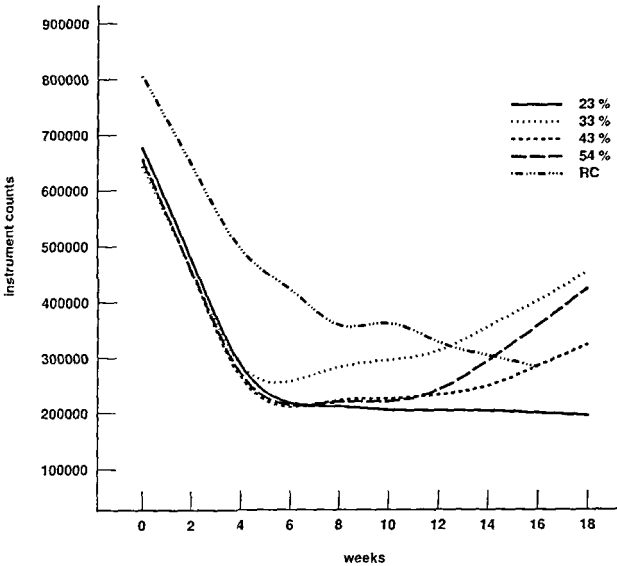


Fig. 4. Variation of Bet v 1 allergen expressed as instrument counts (IC) during the 18-week study period at 20°C at the relative humidities 23%, 33%, 43% and 54%, respectively and in room conditions (rc). Number of measurements: 10 (9 in rc). Weeks 0–18 refer to the time point the samples were taken. Curves were drawn using the mean IC values of three successive biweekly measurements. A single immunoblotting analysis was made for each sample.

ICs towards the end of the 18 week study period (except in the samples of 23% RH and room condition). Similar variation patterns were also observed in the Bet v 1, Phl p 5 allergen and especially birch antigen concentrations. The SQ units varied more irregularly. Towards the end of the study period, birch antigenic activity tended to decrease to the level of onset. Birch and grass pollen antigenic activities intercorrelated in different RH (Table I). The grass pollen sample stored in relative humidity (33%) gradually became moldy beginning in week 4, and by week 20 the pollen sample was infected with mold.

Table I. Spearman's correlation coefficients (*significant coefficients bolded*) for birch and grass pollen antigenic activity after storage at 20°C at different relative humidities (23%, 33%, 43% and 54%, respectively) and in room conditions (rc). Correlations were counted among ICs analysed by IgE immunoblotting and among SQ units analysed by IgG-ELISA using monoclonal (Bet v 1) antibody (mono), polyclonal antibody (poly) and Phl p 5 antibody. N = 17 (16 in room conditions) for birch pollen samples and n = 10 (9 in rc) for grass pollen samples. N = 10 (9 in rc) in immunoblotted samples.

	mono23%	mono33%	mono43%	mono54%	poly23%	poly33%	poly43%	poly54%	IgE-23%	IgE-33%	IgE-43%	IgE-54%	grass23%	grass33%	grass43%	grass54%
33%	0.387 (p=0.125)				0.640 (p=0.006)				0.333- (p=0.347)				0.273 (p=0.446)			
43%	0.502 (p=0.040)	0.321 (p=0.210)			0.610 (p=0.009)	0.907 (p=0.000)			-0.188 (p=0.603)	0.842 (p=0.002)			0.612 (p=0.060)	-0.151 (p=0.676)		
54%	0.463 (p=0.061)	0.047 (p=0.859)	0.248 (p=0.338)		0.654 (p=0.004)	0.659 (p=0.004)	0.650 (p=0.005)		-0.091 (p=0.803)	0.855 (p=0.002)	0.697 (p=0.025)		0.358 (p=0.310)	0.515 (p=0.128)	0.164 (p=0.652)	
RC	0.482 (p=0.059)	0.132 (p=0.625)	-0.094 (p=0.729)	0.603 (p=0.013)	0.479 (p=0.060)	0.397 (p=0.128)	0.456 (p=0.076)	0.697 (p=0.003)	0.583 (p=0.100)	-0.200 (p=0.606)	-0.300 (p=0.433)	0.000 (p=1.000)	0.806 (p=0.004)	0.006 (p=0.987)	0.673 (p=0.033)	0.261 (p=0.467)

DISCUSSION

Birch and grass pollen have been shown to maintain their antigenic activity under experimental conditions until the following pollen season, and allergens could thus exist in indoor environments the whole year round. This presumption is supported by earlier separate findings of antigens in indoor settled dust even in February and March (Dotterud et al. 1997, Dybendal et al. 1989, 1991). The present study has also demonstrated that the allergen concentration in pollen grains kept indoors may remain sufficiently high to cause allergic symptoms. Thus long-lasting exposure and sensitisation to pollen allergens is possible the whole year.

Permanent relative humidities did not seem to have any major effect on the maintenance or liberation of pollen antigenic activity. The activity was not destroyed under any of the conditions tested, even at the highest RH. Activities varied similarly under all conditions. In some cases, correlations were found between the activities and different RH conditions (Table I).

Antigen concentrations varied during the whole one-year study period. At the beginning the antigenic activity of both birch and timothy pollen decreased quickly, as seen most clearly in immunoblotting (Fig. 4). Antigens might have been released from the outer pollen structures. Phl p 5 allergens have been detected e.g. in pollen exine locations (Grote et al. 1994) and plenty of pollen proteins have been localised especially on the surface of birch orbicules and in the aperture regions in late maturation (El-Ghazaly & Grafström 1995). This phenomenon of early-released antigens may be related to the observation of pre-season allergic symptoms. The later increase in antigenic activity may be caused by long-lasting exposure to environmental factors, such as pathogenic microorganisms. The exposure may have increased the amount of the pathogen-related Bet v I variation (Swoboda et al. 1995a, b). The biological significance of the synthetization of Phl p 5 allergens is not known. The decrease and later increase may also be merely a normal genetically determined developmental change.

The Phl p 5 concentration was lower than the birch allergen and antigen concentration but it remained high enough to induce sensitisation. Only in the sample stored at 33% relative humidity was the Phl p 5 concentration much lower than the other samples. This sample was infected early, and the mold ultimately permeated the whole pollen sample. This phenomenon can occur in indoor settled dust, and the growth of mould within the pollen grain (Hutchison & Barron 1997) may thus decrease the antigenic activity of pollen within a few weeks even if no saprophyte-resistant proteins are present.

The alteration of birch pollen antigenic activity analysed by IgE-immunoblotting and the ELISA method tended towards the same direction, although the antigenic activity analysed by the ELISA method showed more sporadic variation. These two methods are not directly comparable, due among other factors to the use of different antibodies.

The main finding of this study is that birch and grass pollen maintains their antigenic activity at a sufficiently high level to be able to cause allergic symptoms all year round. This finding gives rise to certain questions for future study:

how high are the pollen allergen concentrations present in indoor settled dust during the year? In particular how do the pollen allergen particles become airborne, so as to be inhalable in an indoor environment? Information on these questions should be important information for people suffering from allergies.

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