Release of Bet v 1 from birch pollen from 5 European countries. Results from the HIALINE study

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Abstract

Exposure to allergens is pivotal in determining sensitization and allergic symptoms in individuals. Pollen grain counts in ambient air have traditionally been assessed to estimate airborne allergen exposure. However, the exact allergen content of ambient air is unknown. We therefore monitored atmospheric concentrations of birch pollen grain and the matched major birch pollen allergen Bet v 1 simultaneously across Europe within the EU-funded project HIALINE (Health Impacts of Airborne Allergen Information Network).

Pollen count was assessed with Hirst type pollen traps at 10 l/min at sites in France, United Kingdom, Germany, Italy and Finland. Allergen concentrations in ambient air were sampled at 800l/min with a Chemvol high-volume cascade impactor equipped with stages PM>10μm, 10 μm>PM>2.5μm, and in Germany also 2.5 μm>PM>0.12μm. The major birch pollen allergen Bet v 1 was determined with an allergen specific ELISA. Bet v 1 isoform patterns were analyzed by 2D-SDS-PAGE blots and mass spectrometric identification. Basophil activation was tested in an FcεR1-humanized rat basophil cell line passively sensitized with serum of a birch pollen symptomatic patient.

Compared to 10 previous years, 2009 was a representative birch pollen season for all stations. About 90% of the allergen was found in the PM>10μm fraction at all stations. Bet v 1 isoforms pattern did not varied substantially neither during ripening of pollen nor between different geographical locations. The average European allergen release from birch pollen was 3.2 pg Bet v 1/pollen and did not vary much between the European countries. However, in all countries a >10-fold difference in daily allergen release per pollen was measured which could be explained by long-range transport of pollen with a deviating allergen release. Basophil activation by ambient air extracts correlated better with airborne allergen than with pollen concentration.

Although Bet v 1 is a mixture of different isoforms, its fingerprint is constant across Europe. Bet v 1 was also exclusively linked to pollen. Pollen from different days varied >10-fold in allergen release. Thus exposure to allergen is inaccurately monitored by only monitoring birch pollen grains. Indeed, a humanized basophil activation test correlated much better with allergen concentrations in ambient air than with pollen count. Monitoring the allergens themselves together with pollen in ambient air might be an improvement in allergen exposure assessment.

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Introduction

Allergies are the most prevalent chronic disease in Europe with an >20% prevalence (Bauchau and Durham, 2004; Sunyer et al., 2004; Bousquet et al., 2007). Allergic diseases to airborne allergens have been steadily increasing over the past decades (Eder et al., 2006). This increase in prevalence is also due to replacement of older less sensitized individuals in the population by younger individuals with a higher degree of sensitization (Jarvis et al., 2005; Rönmark et al., 2009; Laatikainen et al., 2011). For developed countries a leveling-off of allergic disease prevalence was reported (Asher et al., 2006; Grize et al., 2006), but for some highly developed countries like Finland an end of this epidemic is not in sight (Laatikainen et al., 2011). Allergen exposure determines sensitization (Olmedo et al., 2011) and allergy symptoms correlated with exposure (Corrigan et al., 2005; Brito et al., 2011). Exposure to outdoor airborne allergens is monitored by determining the concentration of pollen in ambient air with a network of over 350 pollen traps spread over Europe (www.polleninfo.org, accessed January 2012). However, humans react to the allergen and the concentration of airborne pollen (the pollen count) is a proxy of exposure. Indeed, several investigations imply that the pollen count might not be representative for allergen exposure, also because allergen was found in non-pollen bearing fractions of ambient air (Schäppi et al., 1997b; De Linares et al., 2010; Fernandez-Gonzalez et al., 2011).
Pollen grains release more immunologically active compounds than only allergen, like PALMS, adenosine and NADPH oxidase (Dharajiya et al., 2007; Gilles et al., 2009; Gilles et al., 2011). These compounds can act as adjuvants, however the allergen from pollen is the dominant factor for evoking symptoms (Brito et al., 2011).
Almost all patients allergic to birch pollen are allergic to Bet v 1, sometimes accompanied by a sensitization to Bet v 2 or Bet v 4 (Moverare et al., 2005). The Bet v 1 content of birch pollen is not constant (Buters et al., 2010), and geographical variation was described (Buters et al., 2008). Climate change with increasing concentrations of CO_2 results in higher pollen production as CO_2 is both an airborne fertilizer and a greenhouse gas. Changes in allergen release per pollen would be in addition to the changed load of airborne pollen (Estrella et al., 2006; Rogers et al., 2006; Shea et al., 2008; Ziska and Beggs, 2012). We therefore determined the variation in the release of the major birch pollen allergen Bet v 1 with an immunochemical ELISA method in the project HIALINE (Health Impacts of Airborne Allergen Information Network) and confirmed this independently in selected cases with a bio-assay using FcεR1-humanized rat basophils. We evaluated whether the used methods, Chemvol® and ELISA, were suited for an allergen-release measuring network. We also investigated whether meteorological factors could govern allergen release from pollen, in an effort to predict the effect of climate change on the allergenicity of pollen.

Materials and methods

Pollen count

Airborne concentrations of pollen were sampled with volumetric spore traps of the Hirst design (Hirst, 1952) and examined by light microscopy as described before (Smith et al., 2009). Pollen counting methods vary historically between countries and three different methods were included in this study. Three stations used a technique where slides were examined along three (France) or four (Germany and Italy) longitudinal transects (Sikoparija et al., 2011). In the UK pollen grains were counted along twelve latitudinal transects (Smith et al., 2009). The analysis method used in Finland is random sampling of microscopic fields which has been shown to give parallel results to the counts carried out with the two other methods (longitudinal and latitudinal transects) (Mäkinen, 1981). Difference between methods was eliminated by correction for surface counted (Comtois et al., 1999). Pollen counts for the 10-years average were obtained from the EAN (European Aeroallergen Network, https://ean.polleninfo.eu, accessed January 2012). The pollen season was calculated as described in the legend of Table 1. The pollen index, the total exposure to pollen from one season at one station was expressed as the sum of daily average pollen concentrations according

$$\int_{t=0}^{t \to end} Ct \bullet d(t)$$

and carries the unit Σ pollen grains/m^3.

Quality control of the pollen count was monitored by the UK partner. All partners filled in questionnaires requesting data on the siting and operation of the pollen trap and the preparation and counting of samples. In addition, calibration slides were sent to each station and everyone involved in counting pollen for the study examined the slides. The limits imposed on the quality control survey were: (1) pollen counts between 0-30 pollen grains/m^3 had to be within ±10 pollen grains/m^3; (2) pollen counts >30 pollen grains/m^3 had to be within ± 30%. The rule that pollen count between 0-30 pollen grains/m^3 had to be within +/-10 pollen grains/m^3 was introduced because very low pollen count can easily vary by more than 30% (Sikoparija et al., 2011). This number (±30%) was also determined by Comtois et al. as the inherent variation of the method (Comtois et al., 1999).
The variability between Hirst type volumetric spore traps was determined with 3 samplers operating simultaneously over a 3-week period at <5m apart on a rooftop at 9m a.s.l. during the birch pollen season in Munich, Germany (n=63, pollen between 0 and 4500 grains/m^3).

Airborne allergen sampling

Air was sampled as previously described (Buters et al., 2010). In brief: 800l/min ambient air was sampled on polyurethane foam with a high-volume Chemvol® cascade impactor equipped with size class stages PM>10μm and 10μm>PM>2.5μm

(Butraco Inc., Son, Netherlands) (Demokritou et al., 2002). In Munich, the stage 2.5μm>PM>0.12μm was also sampled. Air flow was kept constant with a rotameter controlled high-volume pump (Digitel DHM-60, Ludesch, Austria). At each site, the Chemvol® sampler was located at equal height and within 5m of a Hirst-type trap. For each station Chemvol® and Hirst type pollen samples were analyzed daily for identical time periods. Polyurethane foam impacting substrates were cut into 3 identical parts per day and stored at ≤-20°C until extraction.

Meteorological data

Weather data (daily average temperature, daily average humidity, cumulative daily rainfall, average wind speed, and cumulative daily sunshine) were measured using sensors at the stations. Missing parameters were obtained from the closest nearby stations of the national weather services. In the UK this was the Pershore station, in France the station at the airport of Bron, in Germany station 3379, Munich City of the Deutsche Wetterdienst, in Finland it was Turku Artukainen (Airport) weather station. In Italy all was measured at the same location as the Chemvol sampler.

Extraction and analysis of Bet v 1

The Chemvol® polyurethane impacting substrates were extracted for 4h in 0.1M ammonium bicarbonate pH8.1 in a head-over-head rotator. Extracts were aliquoted, frozen, lyophilized and stored at ≤-20°C. Aliquots were reconstituted in 0.1M phosphate buffered saline pH7.4, serial diluted and allergen was determined by using a 2-site binding assay based on monoclonal antibodies in an ELISA format. Monoclonal antibodies 4B10 and 2E10 specific for Bet v 1, natural purified Bet v 1 standards and controls were provided by our partner Allergopharma KG, Reinbek, Germany (Chapman et al., 2008; Kahlert et al., 2008).

With each ELISA two control samples of different concentration were analyzed. The values of these controls had to be within 25% of a reference value for the ELISA to be accepted. Then only those values of serial dilution that yielded the same concentration were reported.

For each day at least two filter parts were independently analyzed. If two filter parts did not yield a value within 25% of each other, a third extraction was performed and analyzed. The final reported concentration of each day was the mean of all valid determinations, mostly the mean of at least 16 ELISA wells. The same Standard Operating Procedure (SOP) was used by all partners, which included written data inclusion rules.

Bet v 1- isoform analysis

Pollen was sampled sequentially from several locations across Europe (not always the stations were the allergen measurements were done) as described previously by Buters et al. (Buters et al., 2010) and shipped at -20°C to our partner at Allergopharma. Pollen from the day of pollination of each *Betula pendula* tree were extracted (see above) and subjected to 2D-SDS-PAGE electrophoresis using a first dimension of isoelectric focusing between pH4-7 (IPG strips, GE Healthcare, Munich) and a second dimension of SDS-PAGE (Excel SDS gel, 12 – 14%, GE Healthcare, Munich) for size separation. Gels were stained with Coomassie Brilliant Blue, blotted and dried. Available recombinant Bet v 1.0401 (Bet v 1d) and Bet v 1.0601 (Bet v 1f) were a kind gift of Prof. F. Ferreira, University of Salzburg, Austria, recombinant Bet v 1.0101 was from Allergopharma. Spots were quantified using Proteomweaver software (Definiens, Munich, Germany) and expressed as relative % of the sum of all intensities. Punched spots were identified by tryptic digestion and analysis by mass spectrometry as described by Sarioglu et al. (Sarioglu et al., 2008).

Degranulation of humanized RBL

The humanized rat basophil cell line RBL-2H3 clone h21 expressing the α, β, and γ chain of the human FcεR1 was a kind gift of Prof. S. Vieths, Paul Ehrlich Institute, Langen, Germany. Cells were grown under standard conditions (Vogel et al., 2005) and were passively sensitized with serum of a birch pollen symptomatic patient (skin prick test positive and RAST>3). Dilutions of daily Chemvol samples were added to the cells and degranulation was quantitated as ß-hexosaminidase release, determined as nitrophenol release from pNAG (p-nitrophenol-D-2-acetamido-2-deoxyglucopyranosid, Sigma-Aldrich Corp, St. Louis, MO) in relation to total ß-hexosaminidase activity after lysis of the cells with 1% Triton-X100 (Vogel et al., 2005). Only values within the linear dose-response range of the cells (5-45% degranulation) were reported. Because extracts vary greatly in Bet v 1 content, degranulation was calculated as if 1m^3 air was given to the cells. This could result in hypothetical degranulations of up to 800%.

Modeling

The System for Integrated modeLling of Atmospheric coMposition (SILAM, (Siljamo et al., 2008a)) was used to compute the footprints of the observations and also to evaluate the flowering season.

A footprint of a single observation is, by definition, a surface area that delineates the sources that are responsible for the observed atmospheric concentrations. Interpreted in probabilistic terms, the footprint shows the probability of a specific air parcel to reach the observational site. The areas, for which this probability is not zero, comprise the footprint of the particular observation. As a simplification, all sources located within the footprint area would influence this observation, and no sources located outside the footprint area would affect it. The specific contributions of these sources vary depending on the footprint value: the higher the value, the stronger the source impact. Computations of the footprint with standard dispersion models are prohibitively resource-consuming, while the adjoint modeling used in this study generates the solution with reasonable efforts (Sofiev et al., 2006b).

The flowering season prediction followed the thermal-type model as described before (Sofiev et al., 2006a; Siljamo et al., 2008b). The SILAM model was run with a time step of 15 minutes and evaluated the transport for 60 hours backward in time, for each daily observation at each site. The configuration included 8 vertical layers up to ~6 km above the ground. The horizontal grid cell size was 25 km and the domain of simulations covered almost the whole of Europe. Meteorological information was taken from the operational archives of the European Centre of Medium Range Weather Forecast (ECMWF). This data had a spatial resolution of about 25km and time step of 3 hours.

Statistical analysis

Differences were analyzed with a paired Student's *t*-test unless stated otherwise (Livingston, 2004). A p value <0.05 was considered statistically significant. Outliers were defined as more than 3 standard deviations of the mean. The relationship between allergen and pollen count was presented graphically by scatter plot. The strength of the relation was expressed by the coefficient of correlation (r^2) which was calculated by using linear regression (Lorenz, 1989). The same pre-given spreadsheet was used for all calculations.

Results

Pollen count

Although the pollen counting method differed between the stations, this was compensated by correcting for the counted surface area and reporting the number as pollen grains/m^3. This is a common method also used by EAN. Indeed, when we counted the same slides for birch pollen both latitudinal or longitudinal (n=15) in the same laboratory by the same operator this resulted in a <7% difference, in agreement with the literature for other pollen species (Carinanos et al., 2000).
In our network out of a total of 28 calibration counts for *Betula*, three were outside the limits imposed on the Quality Control survey (11%).
The variability of pollen counts at the same location between 3 Hirst type pollen traps was 23%. Recounting pollen from the same slide (n=8) by the same operator showed a <4% variability in counting reproducibility, the same as reported before (Kapyla and Penttinen, 1981). Thus 19% of the variation in birch pollen count is due to differences between the samplers.

The annual sum of pollen (pollen index, see methods) in 2009 varied between 235 Σ pollen grains/m^3 in Italy to 3144 Σ grains/m^3 in Germany (see Table 1). The average annual birch pollen count in 2009 was 55% of the 1999-2009 average birch pollen flights for these stations (range 30 to 90%, see Table 1), and similar years did occur for each station (data not shown). Thus the birch pollen season in 2009 was representative for all stations. Other European stations (not in this manuscript) report higher 10-year average counts for birch pollen (i.e. central Finland, Poland or Ukraine), as the center of birch tree habitat is the eastern part of Europe just outside the European Union (www.polleninfo.org, accessed January 2012). Thus our results cover the extremes of the European Union habitat for birch trees.

Airborne pollen allergen

The detection limit of the ELISA was 0.7 ng Bet v 1/ml, which equals 2.1 pollen/m^3. Running a high and a low control concomitant with each run monitored the performance of the Bet v 1-ELISA. Data inclusion rules were installed. The variability of the ELISA (n=66, all stations, see Table 2) of the low control (1.9 ng/ml) was 17.4% and 12.8% for the high control (7.4 ng/ml), in close agreement with the literature (Schäppi et al., 1996; Buters et al., 2010). The pollen count and allergen Bet v 1 concentrations are depicted in Figure 1. Allergen was 89.6±1.5% found in the PM>10μm fraction, the remainder was in the 10μm>PM>2.5μm fraction, and none in the smallest 2.5μm>PM>0.12μm fraction, available only in Munich. No allergen was found when no pollen was detected (see Figure 1). The European average Bet v 1 release per pollen was 3.2 pg Bet v 1/pollen (r^2=0.714, see Figure 2). However, the difference in allergen release per pollen between days and locations was >10-fold, even if we deleted all pollen count <10 pollen/m^3 to avoid high allergen release values due to less reliable (low) pollen count. When we removed (arbitrarily) the Bet v 1 release values per pollen stemming from pollen count <10 pollen/m^3, then the average allergen release per pollen of the 10% lowest values was 0.61 pg Bet v 1/pollen, the average of the highest 10% released 8.76 pg Bet v 1/pollen. Within each station, the average allergen release of the highest 5% and the lowest 5% values also varied at least >10-fold (see Figure 2). Between countries the average allergen release per pollen were considered similar as the observed differences are within the uncertainties in pollen count (method variation <30%) or ELISA determination (method variation <17%).

Bet v 1 isoforms

Bet v 1 is increasingly expressed during ripening in the last week before pollination (Buters et al., 2010). The pattern of Bet v 1 isoforms during ripening was determined using one tree, (see Figure 3). Spots of April 18^{th} were identified using mass spectrometry and, if available, by using pure recombinant isoforms.

The isoform pattern of Bet v 1 did not differ markedly during ripening, (see Figure 3). Thus the same fingerprint of isoforms is expressed at rising concentrations during pollen ripening. An exception is spot 1, which was analyzed as being a truncated isoform of Bet v 1.0101 (Bet v 1a), which increases upon ripening of the pollen. However, this was a minor Bet v 1 isoform.

The ELISA antibody combination recognized all isoforms equally as pooled human serum. The ELISA antibodies did not recognize the isoform Bet v 1.0401 (Bet v 1d), which was also not recognized by pooled human serum from 10 donors (data not shown) (van Ree et al., 2008). Our ELISA thus represents human reactivity.

The isoform pattern of birch pollen from several different locations across Europe varied marginally, see Figure 4. Because ripening of the pollen does not influence the isoform pattern, harvesting at not exactly the same time points before pollination would not explain differences. We conclude that the Bet v 1 isoform pattern is the same during ripening and the same across Europe. Our antibodies recognized all isoforms except the hypoallergenic isoform Bet v 1.0401, like humans (data not shown) (see Figure 3) and the difference in Bet v 1 content between the stations is thus due to differences in amount of released Bet v 1, not due to release of different isoforms.

Degranulation of basophils

Rat basophils carrying the human FcεR1-receptor were passively sensitized with serum of a birch pollen sensitive individual and incubated with daily extracts of Chemvol samples from Munich, Germany. The reactivity of the cells is depicted in Figure 5. Again, degranulation was only seen when pollen were counted. In addition, β-hexosaminidase release correlated well (r^2=0.95, insert in Figure 5a) with Bet v 1 concentration in ambient air, but less well with pollen count from Munich (r^2= 0.71, see insert in Figure 5b). The level of detection, defined as 10% degranulation above baseline, was 0.2 ng/ml Bet v 1.

Modeling

For this analysis, due to uncertainties in pollen counts and allergen determination, we omitted pollen counts below 15pollen/m^3 and allergen content below 10pg/m^3. Above this values the measurements are more robust. Analysis of the time series of the allergen release per pollen grain shows several features: (i) an established value for allergen release per pollen, which is steadily between 2 and 4pg/grain during the whole season for all stations, (ii) small fluctuations around this value, which are probably due to uncertainty of both Bet v 1 and pollen observations, (iii) several episodes at some stations lasting for a few days, during which the allergen content differs from the average level by several-fold.

By joint analysis of the observation footprints and flowering patterns, the allergen content during the multi-day episodes can be correlated with geographical location of the pollen sources, as shown in Figure 6. The allergen content observed in Turku is systematically low during the period 12 - 17 May, with the gradual decrease during 12-15 May down to less than 1 pg/pollen and then increase again during 15-17 May. The footprint analysis showed that the transport direction of pollen was gradually changing from central Sweden to northern Finland and then further to southern Finland. All these areas were flowering during these days. This suggests that the pollen originating from northern Finland showed an about three-times lower allergen release than that from the more southern regions. A similar pattern was seen again 24 and 25 May when the footprint was covering northern Sweden, which was flowering at that time (not shown). There was again a 3 times lower allergen release in pollen from the north than during the days when pollen originated from more southern regions.

In Munich, the episode during April 16-18 was characterized with high allergen content, flanked before and afterwards by several days with an average allergen release per pollen. Before and after that period the footprint shows the source areas to the east and somewhat to the south of Munich and the allergen release was at the average level. However, during the episode, the pollen mainly originated from mountains of Switzerland, where birch was at full flowering. Those grains had 2-3 times more allergen release than regions to the east of Munich. The part of footprint covering the Alps was probably void because there was no flowering in the high mountains yet.

Discussion

Birch pollen and the major birch pollen allergen Bet v 1 were sampled during the birch pollen season in France, United Kingdom, Germany and Finland (and some data from Italy). The birch pollen season in 2009 was a representative year for all stations.
We did not find any large difference in Bet v 1 isoform patterns during ripening of the pollen or across Europe, except for a minor isoform (truncated Bet v 1.0101, less than 5% of total Bet v 1). The isoform pattern recognized by ELISA was identical to the pattern recognized by a serum pool of birch allergic individuals. The average Bet v 1 release per pollen from the different stations was within the error of the methods for pollen counting (30%) and ELISA (17%) and we consider the Bet v 1 release from pollen in 2009 similar across Europe. However, daily Bet v 1 release varied >10-fold in all countries. This was independently confirmed with a bio-assay for Bet v 1 using human sIgE in humanized rat basophils. Indeed, ß-hexosaminidase release in these cells as a proxy for histamine release correlated better with Bet v 1 than with pollen concentrations.
We conclude that birch pollen can vary >10-fold in allergen release and that this variation is similar across Europe.

Pollen count

Although pollen counts in Europe have been performed using standard methods for decades (Hirst, 1952), little is known about the reproducibility of Hirst type volumetric spore traps (Barral et al., 2009). In Munich, birch pollen count recorded by three volumetric pollen traps simultaneously varied by 19% for birch pollen. Comparing the different pollen counting methods (longitudinal, latitudinal and random) by sending calibration slides to all stations showed that the majority of birch pollen count (89%) varied by less than 30%, in agreement with previous publications (Comtois et al., 1999; Carinanos et al., 2000; Sikoparija et al., 2011).
Comtois et al. suggested that there will always be imprecision linked with the airborne pollen count unless aerobiologists count the whole slide, and even then variation between pollen traps will still be present. There is always a trade off between precision and the amount of time required to produce the daily pollen count (Comtois et al., 1999).
Based on these results and the literature we believe that a variation of <30% in our data for pollen count is already accounted for by the pollen sampling and counting methodology and is not due to variations in nature.
The pollen season in 2009 was a representative year for all stations, as the average annual sum of birch pollen in 2009 was 55% (range 30-90%) of the 10-year average for each station with no outliers (see Table 1). The stations also cover a larger geographic area within Europe.

Allergen Bet v 1 in ambient air

The pH of the extraction buffer is likely to influence the amount and isoform pattern of the allergens extracted. We used a slight alkaline extraction buffer, a condition observed in nasal fluid of allergic patients (Podoshin et al., 1991) and recommended by Cadot et al. for the appearance of relevant isoforms (Cadot et al., 1995). Indeed, extraction at pH8.1 was used by several, but not all authors (Rantio-Lehtimäki et al., 1994; Schäppi et al., 1997b; Petersen et al., 2001).

The average Bet v 1 release across Europe was 3.2 pg Bet v 1/pollen and is similar to the value of 4 pg Bet v 1/pollen reported before (Schäppi et al., 1997b). Schenk et al. also published that the variation in allergen release between birch species was limited (Schenk et al., 2011) but report higher allergen content per pollen, which could be due to their extraction method. Another difference with other authors is that in the current study pollen were sampled from ambient air and not from trees or commercial suppliers.

The average Bet v 1 release per pollen per country did not vary much (-23.1% in the UK and +21.8% in Germany, see Figure 2) and could be explained by variations in methodology as we determined that between pollen samplers a variation of 23% existed and our ELISA has a <17% variation between laboratories in agreement with the literature where 30% variations for pollen monitoring is reported (Comtois et al., 1999; Sikoparija et al., 2011) and similar variations were found for ELISA allergen determinations (Schäppi et al., 1996; van Ree et al., 2008).

However, when comparing the 10% lowest allergen release values per pollen with the 10% highest values, the daily allergen release difference across Europe and also within each country was >10-fold. Several other authors also report differences in allergen release per pollen for birch and olive pollen (Pehkonen and Rantio-Lehtimäki, 1994; Schäppi et al., 1997a; De Linares et al., 2007; Buters et al., 2010; Brito et al., 2011). This study shows that the variation in allergen release per pollen is substantial but equally distributed across the European birch tree habitat.

Across Europe, about 90% of the allergen was recovered from the >10μm fraction, none in the 2.5μm>PM>0.12μm fraction (only in Munich), and no allergen was detected when no pollen was detected, neither with our ELISA nor with a more sensitive bio-assay. This shows that the only source of allergen is birch pollen, in agreement with previous results where allergens were monitored for several years on a row at one location (Buters et al., 2010). Birch allergen containing particles were reported in the fractions PM<10μm (birch pollen have a geometric diameter of 21-24μm (Brown and Irving, 1973; Rantio-Lehtimäki et al., 1994; Schäppi et al., 1999)) indicating in combination with our results that if such particles exist, their appearance is rare and might need specific atmospheric conditions like thunderstorms (D'Amato et al., 2008), which did not occur during our experiments.

The role of Bet v 1 isoforms

We found that the Bet v 1 isoform fingerprint was more or less constant during ripening and across Europe with minor variations (see Figures 3 and 4), in agreement with other authors (Friedl-Hajek et al., 1999; Erler et al., 2011; Schenk et al., 2011). The isoform truncated Bet v 1.0101 did increase in expression upon ripening of the pollen, however this minor truncated Bet v 1.0101 isoform represented <5% of total Bet v 1 isoforms. The identity of the spots was confirmed with LC-MS after tryptic digest (Sarioglu et al., 2008), and if possible, by comparison with recombinant proteins. Also, our antibodies did not recognize the hypoallergenic isoform Bet v 1.0401 (Bet v 1 d), similar to a pool of human serum (data not shown) and as reported by others (Friedl-Hajek et al., 1999; Erler et al., 2011). Thus the difference we measured must be due to different total concentrations of allergenic Bet v 1 of which 50-70% is Bet v 1.0101(Bet v 1a) (Erler et al., 2011).

Clinical relevance

We used an independent method from ELISA to determine allergen content in ambient air by taking human serum from a birch pollen sensitive individual and passively sensitizing basophils (see Figure 5). Although human mast cells would be the preferred cells, few human mast cell lines are available and none has been shown to be suitable for allergen determination (Kirshenbaum et al., 2003; Guhl et al., 2010). Besides mast cells, basophils are also responsible for the clinical symptoms of allergy in humans (Gibbs, 2007). The basophil cell line with human Bet v 1 specific IgE as detector of environmental Bet v 1, resulted in an immune response as seen with allergic individuals (Vogel et al., 2005). Although more tedious, this method is more sensitive than the ELISA, able to detect 0.2 ng Bet v 1/ml (defined as degranulation >10%). With this more sensitive bio-assay also no allergen was detected when no pollen were measured. Also, β-hexosaminidase release (a substitute marker for histamine release which is the hallmark of allergic disease), correlated well with Bet v 1 ambient concentrations (r^2=0.95), but lesser good with pollen count (r^2=0.72, see Figure 5).

Modeling

We found several multi-day episodes where the differences in pollen potency could be explained by differences in origin of the pollen (see Figures 1 and 6). We omitted pollen counts below 15 pollen/m^3 and allergen measurements below 10 pg/m^3 to guarantee more robust data. We also focused on multi-day episodes, as one-day jumps of the pollen content are more difficult to analyze. In general, there can be three possible explanations for single day jumps:

(i) similar to multi-day episodes, the peaks may correspond to specific transport conditions and/or origin of the grains. However, footprint analysis did not reveal such dependence.

(ii) low pollen count means higher uncertainty of the allergen release per pollen. However, it should manifest itself as both anomalously high and low values, which is not the case: almost all low-count cases were characterized by the high allergen release.

(iii) there can be allergen present in air apart from the one encapsulated in the pollen grains (D'Amato et al., 2008). Even when the number of grains is low, this extra allergen could lead to high release estimates. The instrumentation used in the current study does not detect such allergen, thus does not allow an explicit check of this possibility. Using suitable equipment, no such free allergen was detected (Buters et al., 2010).

Several multi-day episodes were detected (see Figure 6). They corroborate our finding with ELISA and the bio-assay that pollen is not constant in their allergen release.

Noteworthy, the average value also suggests some north-to-south gradient: for Munich and Worcester the allergen release is about 3 pg/pollen, whereas for Turku it is about 2 pg/pollen (statistically non significant).

Conclusions

In daily samples taken during the birch pollen season in 5 European countries we could only detect allergen when pollen was present. Also allergen was predominantly found in the PM>10μm fraction, the fraction where the pollen land in the cascade impactor. Thus Bet v 1 in ambient air was confined to birch pollen. With our method the average European allergen release per pollen was 3.2pg Bet v 1/pollen. The average allergen release in 2009 did not vary substantially between countries. However, a >10-fold difference between daily allergen release per pollen was detected in all countries. Thus pollen exposure qualitatively represents allergen exposure but not quantitatively. The allergen concentration also correlated better with the bio-assay for immune response than pollen concentration. Modeling showed that multi-day episodes exist were pollen from specific origins consistently varied in allergen release. Thus we expect allergen monitoring to be a more accurate predictor of human allergic symptoms than pollen count.

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Legend to the Figures

Figure 1. Daily values in 2009 for birch pollen (grey bars) and Bet v 1 (colored lines) in PM>10μm (green) and 10μm>PM>2.5μm (red) from the different European stations. (A) Munich, Germany (B) Turku, Finland (C) Lyon, France (D) Worcester, UK. Only Munich, Germany additionally sampled 2.5μm>PM>0.12μm (yellow). Note: the scales differ between stations for clarity. The amount of daily allergen released per pollen is given for pollen counts >10 pollen/m^3 (pink).

Figure 2. Correlation between the total releasable allergen in the air and pollen count in the different countries in 2009. The slope of each linear regression curve represents the average Bet v 1 release per pollen for that country. Each point represents duplicate determinations of one day in one country. - ● - Germany; - ■ - Finland; -▲- France; - ▼ - United Kingdom; - ♦ - Italy. The data point indicated with the arrow was treated as an outlier.

Figure 3. One D (A) and 2D (B) SDS-PAGE blots of 0.1 M ammonium bicarbonate extracts from pollen harvested sequentially in 2008 from one tree in Germany. April 19-20 were days of maximum pollination of that tree. Lane A is the image of the blot of the molecular weight marker (15kDa) and pollen extract after separating according to size before development into the second pH4-7 dimension in the right lanes. Identity of the spots was determined for April 18th. Spot 0101 represents Bet v 1.0101(Bet v 1a), the others are Bet v 1.0401(Bet v 1d), Bet v 1.0601 (Bet v 1f), Bet v 1.1401 (Bet v 1m) and truncated Bet v 1.0101(t0101, see methods).

Figure 4. Isoforms of Bet v 1 from trees across Europe in 2009. The same methodology as for Figure 3 is used.

Figure 5. ß-hexosaminidase release (a proxy of histamine release) of FcεR1-humanized RBL cells after passive sensitization with serum of a birch pollen sensitized individual (see methods) and exposure to daily samples of TUM2009. Samples were diluted to fit the dynamic range of the cells. Degranulation per cubic meter air was then calculated and depicted and may exceed 100%. (A) Bet v 1 concentrations and ß-hex- (B) Pollen concentration and ß-hexosaminidase release.

Figure 6. Flowering of birch trees (pink) and the observation footprint (area where the particles collected in the instrument stem from considering the last 60hrs, blue). Date, pollen concentration and their potency (pg Bet v 1/pollen) is given. The potency of pollen can depend on the area of origin. (A) Turku, Finland and (B) Munich, Germany.

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