Arch Immunol Ther Exp, 2005, 53, 169–174
PL ISSN 0004-069X

Received: 2004.08.03
Accepted: 2005.12.15
Published: 2005.04.15

Quantification of airborne birch (*Betula* sp.) pollen grains and allergens in Krakow

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Jacek Madeja[1ABDEF], Ewa Wypasek[2ABDEF], Barbara Plytycz[2DEG], Krzysztof Sarapata[3C] and Krystyna Harmata[1BDG]

[1] Institute of Botany, Jagiellonian University, Krakow, Poland
[2] Institute of Zoology, Jagiellonian University, Krakow, Poland
[3] Department of Bioinformatics and Telemedicine, Medical College, Jagiellonian University, Krakow, Poland

Source of support: grants DBN-414/CRBW/K-VI-2/2003, DBN-25/CRBW-VI-24/2002, and KBN 2 P04G 019 26 to Dr. K. Harmata and by IZ/DS/ZIE.

Summary

Introduction: Birch (*Betula* sp.) pollen grains are the main cause of seasonal allergies in northern and central Europe. The allergen particles released from the grains are often well distributed in the air. Due to their size, airborne protein particles can easily penetrate into the lower parts of the respiratory airways and may lead to symptoms of asthma. The purpose of this paper was to quantify both *Betula* sp. pollen grains and allergens in the air.

Materials and Methods: Materials for the investigation were collected in the spring of 2003 with two Hirst-type pollen volumetric traps. Tapes from one trap served for routine birch pollen grain counts, while those from the second for the immunodetection of birch allergens. As birch pollen allergen concentration is seen as dark spots on X-ray films densitometric measurements of the spots were used to quantify birch-pollen antigen concentrations in the air.

Results: In most instances, birch pollen counts corresponded with birch pollen allergen levels. However, on several occasions outside the pollen season, only grains or only allergens were detected. Apart from sampling variability, this could be due to faulty/dead pollen grains or submicronic airborne allergen particles.

Conclusions: Counting intact pollen grains and antibody-based detection of allergen molecules are efficient tools in controlled allergen avoidance.

Key words: **pollen allergy • pollen allergen release • immunodetection • pollen traps • birch allergens • *Betula***

Full-text PDF: http://www.aite-online/pdf/vol_53/no_2/7153.pdf

Author's address: Barbara Plytycz, Department of Evolutionary Immunobiology, Institute of Zoology, Jagiellonian University, R. Ingardena 6, 30-060 Krakow, Poland, fax: +48 12 634 37 16, e-mail: plyt@zuk.iz.uj.edu.pl

INTRODUCTION

The incidence of certain allergic diseases is increasing in most industrialized countries. This is especially striking in type I atopic diseases such as extrinsic bronchial asthma, allergic rhinoconjunctivitis ("hay fever"), and atopic eczema/dermatitis, but a similar trend is exhibited by cytotoxic (type II), immune-complex (type III), and delayed-type (type IV) hypersensitivity reactions. Various hypotheses have been suggested to explain the causes of the prevalence of certain allergies in certain "westernized" countries during the past few decades and it was concluded that perhaps several factors characteristic of a "modern society" act together[18]. As discussed by Ring et al.[18], these include increased awareness of the diseases, improved diagnostics, genetic susceptibility, psychosocial influences, allergen exposure, decreased immune system stimulation, underlying diseases, anti-allergic therapy, and pollution. The allergenic potency of some causal factors (e.g. pollen allergens) may be enhanced by the adjuvant activity of some biological or chemical factors, especially urban air pollutants due to urban traffic emissions. Conversely, some protective inhibitory factors may be lost due to, for example, decreased stimulation of the human immune system because of improved hygiene (the "hygiene" hypothesis) or vaccination programs[18, 20].

The pollens of trees, grasses, and weeds are the main cause of seasonal allergy in northern Europe[27], which is why pollen counts and pollen-count forecasts are made available[2]. However, pollen allergens have to be released from the pollen grains in order to become allergic. Allergen liberation is an active mechanism dependent on ambient temperature, hour of the day, and relative humidity. Environmental factors, such as high air humidity and thunderstorms followed by dry and sunny weather, facilitate allergen liberation and can explain asthma attacks after rainfall[2, 6, 7]. Moreover, in highly polluted regions wind-borne pollen grains interact with pollutants in a complex manner which modulates allergen release and allergenic potency. For example, SO_2 has an inhibitory effect on allergen release, which contributes to the decreased allergy prevalence in regions polluted by this gaseous pollutant. However, other gaseous pollutants, such as NO_2, do not exhibit these effects. Other pollutants can enhance allergen release and the generation of allergenic aerosols[2]. For these reasons, allergen bioavailability should be considered more thoroughly.

Birch (*Betula* sp.) pollen is an important airborne allergen and is the main cause of the seasonal allergies of 10–20% of the population of northern and central Europe[26]. Birch pollen is also responsible for cross-reactive oral allergies to fruits, nuts, and vegetables. The major *Betula verrucosa* Ehrh. pollen allergen, Bet v 1 (17.4 kDa), is recognized by more than 95% of birch pollen allergic patients, 60% of them reacting exclusively to this allergen[11, 24]. Bet v 1 is found in almost all (>98%) Finish, Swedish, and Austrian sera[14]. Bet v 1 has been extensively characterized, and Bet v 2, Bet v 3, Bet v 4, Bet v 5 have also been cloned and sequenced. Recombinant Bet v 5 has a sequence identity of 60–80% to isoflavone reductase, a protein family (homologues in various plants) that is involved in plant defense reactions. Bet v 5 may be responsible for pollen-related oral allergy to specific foods in a minority of patients who also have birch pollen allergy[12].

The release of submicronic protein particles from *Betula* sp. pollen grains, which can penetrate into the lower parts of the respiratory airways, can lead to serious symptoms of asthma, besides rhinitis. The allergen particles are often well distributed in the air and their concentration might be high even when the pollen grain concentration is relatively low[17]. Due to this fact it seems important to monitor the air concentration of pollen allergens rather than that of pollen grains.

Birch trees (*Betula* sp.) are common throughout Poland[28]. Consequently, birch pollen is very abundant in the air during the flowering season. Two *Betula* sp., *B. verrucosa* Ehrh. and *B. pubescens* Ehrh., are sub-constituents of forests outside of the city of Krakow. *B. verrucosa* Ehrh. is also commonly grown as an ornamental tree in the town parks and along some streets (personal observations). In Krakow (southern Poland), airborne pollen grains and spores have been monitored since 1982[22]. The purpose of this paper was to quantify for the first time both *Betula* sp. pollen grains and allergens; the latter detected by immunoblotting and chemiluminescence.

MATERIALS AND METHODS

Collection of airborne birch pollen samples

The sampling was performed using two volumetric Hirst-type pollen samplers[8] placed on the roof of a 20-m-high building in the center of Krakow (50°03'N, 19°57'E). Standard monitoring of pollen and spore concentration was performed with a Hirst--type Burkard Seven-Day Pollen and Spore Trap (Burkard Manufacturing Ltd., Rickmansworth, Hertfordshire, UK). The birch pollen grains were identified on the basis of their characteristic shape and size[13] and counted in equidistant vertical zones

corresponding to 2-h periods. The grain counts on the Burkard tape served to estimate the start and the end of the local birch flowering season by the so--called "97.5% method"[16]. A Hirst-type Lanzoni VPPS 2000 trap (Lanzoni S.R.L., Bologna. Italy) was placed next to the Burkard trap and was used for the immunochemical analysis described below. The sampling started on March 10, 2003 (before the onset of the birch flowering season in Poland) and finished on May 27, 2003 (after pollination in the Krakow area).

Immunoblotting and chemiluminescence

Immunoblotting of allergens from the trap to the blotting membrane was performed according to a method adapted from Ekebom's et al.[4] procedure. After every sampling, the Lanzoni tape was cut in 24--h segments. Polyvinylidene difluoride (PVDF) blotting membranes (0.2 μm Biotrans™ PVDF Membranes, ICN Pharmaceuticals, Inc., USA) were soaked for 5 min in methanol before use, rinsed in distilled water for 5 min, and then saturated with transfer buffer for an additional 5 min. The protein particles from the sampling tape were transferred onto PVDF membranes so prepared by 30 min capillary blotting. Another part of the PVDF membrane was used for preparation of the standard curve of *Betula* sp. pollen allergen concentration. For this purpose, dot blots were obtained by serial dilutions of 10 μl/dot of *B. verrucosa* Ehrh. pollen extract, kindly provided by ALK-Abelló (Hørsholm, Denmark). After blotting, the membranes were placed in blocking solution (fat free 5% milk in TTBS solution) overnight. On the following day the membranes were washed in TBS, TTBS, and again TBS for 15 min in each solution. The membranes were then incubated for 40 min with polyclonal antibodies against *B. verrucosa* Ehrh. pollen antigens produced in rabbit (kindly donated by ALK-Abelló, Hørsholm, Denmark) diluted 1:10,000 in TBS solution. After washings in TBS, TTBS, and again in TBS solutions (15 min each), the membranes were incubated with goat-anti-rabbit IgG conjugated with horseradish peroxidase (ICN Pharmaceuticals, Inc., USA) diluted 1:5,000 in TBS solution for 40 min followed by repeating the above 3 washings.

The next steps were performed in total darkness. First the membranes were incubated with chemiluminescence ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, USA) for 5 min. Then excess reagent was absorbed by filter paper and the membranes were sealed in thin plastic wrap. Next they were inserted into the exposure cassette and covered with a sheet of Kodak BioMax MR film. After 5 min exposure, the film was developed according to the directions given by Kodak. Light emitted at the sites of *Betula* sp. allergen was visualized as black or gray spots on the film. Densitometric measurements of spots on the film were performed with a Fluoro-S™ MultiImager (BioRad, USA) and expressed as optical density units.

Statistical analysis

After verification of the empirical distribution of the data sets (rejection of the normal distribution), the Spearman Rank correlation coefficient of pollen grain number and allergen concentration was calculated (non-parametric statistical correlations). Analyses were performed using statistical software package Statistica (version 6.1).

RESULTS AND DISCUSSION

The quantification of airborne birch pollen antigens by immunoblotting and chemiluminescence was originally used by Ekebom et al.[4]. They used a 0.45 μm PVDF membrane, overnight blockade by 4% BSA, secondary antibodies conjugated with alkaline phosphatase, product of reaction detected by a Lumi-Phos 530, and the emitted light measured by a luminometer. Our modifications consisted of a more sensitive 0.2 μm membrane, overnight blockade by 5% skimmed milk, HRP-conjugated secondary antibodies, and an ECL detection system followed by X-ray film exposure and densitometry. The resulting light intensity[4] and dark spots (in our studies) gave good quantitative relationships with protein concentrations of serially diluted birch pollen extracts from ALK-Abelló, with regression coefficients of 0.95–0.99[4] and 0.98 in our system (Fig. 1). Thus, the sensitivity of the method used here is fully comparable with the original method of Ekebom et al.[4]

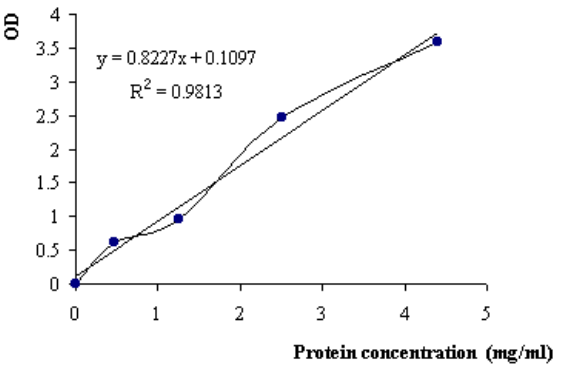

Figure 1. Densitometric measurements (in optical density – OD units) of the dark spots on films exposed to chemiluminescent light originating from secondary antibodies bound to various concentrations of *Betula verrucosa* Ehrh. pollen allergen extracts (mg/ml). The linear regression coefficient was 0.98. R^2 – regression coefficients.

The Nilsson and Persson's method[16] applied for counting birch pollen grains in the Burkard trap (collected from March 10th to May 27th) suggested that in 2003 the local *Betula* sp. flowering season began in Krakow on March 28th and ended on April 29th (Fig. 2).

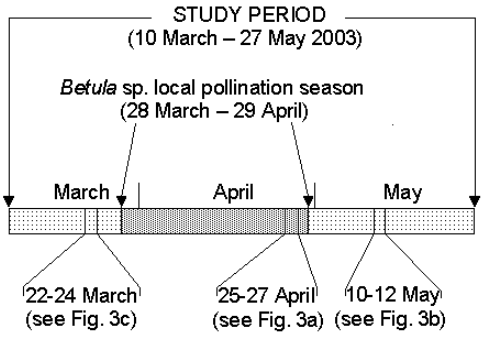

Figure 2. Time scale of the observation period and the estimated local pollination season of *Betula* sp. in Krakow in 2003. The results obtained during the periods indicated on the bottom are illustrated in Fig. 3.

Representative results of the quantification of birch pollen grains and allergens collected by Burkard and Lanzoni traps in Krakow are given in Fig. 3. Birch pollen allergen concentrations, which are documented by dark spots on films, show allergen sites on the tape from the Lanzoni trap. Spot concentrations varied considerably during the period of investigation (see top rows of Figs. 3a–c), and were converted to the densitometric data shown in hourly intervals on the bottom of Figs. 3a–c (right axis). The same bottom rows of Figs. 3a–c illustrate the numbers of pollen grains collected in the same hourly intervals on the tape from the Burkard trap (left axis). Three types of interdependencies of birch pollen allergens and birch pollen grains are illustrated in Fig. 3. First, presence of both pollen grains and allergens (Fig. 3a); second, presence of pollen allergens and absence of grains (Fig. 3b); and third, presence of pollen grains and absence of allergens (Fig. 3c).

The first type of birch pollen-allergen dependency was the most frequent during the local *Betula* sp. flowering season. Good quantitative relationships are visible in Fig. 3a, where the high pollen grain number recorded on April 25 corresponded with the highest allergen concentration, while on April 26 the low grain number was accompanied by the small amount of allergen ($r=0.9504$; $p=0.0000$).

The detection of birch allergens on the tape from the Lanzoni trap when there were few or no pollen grains on the tape from the Burkard trap happened on several occasions during the whole observation period. This is illustrated by the records from 10–12 May, i.e. after the birch pollination season ($r=0.1619$, $p=0.4497$; Fig. 3b). The opposite situation, namely the presence of birch pollen grains in the Burkard trap without birch pollen allergens in the Lanzoni trap, was seldom and recorded only before the *Betula* sp. flowering season, e.g. on March 22–24 ($r=0.6421$, $p=0.0007$; Fig. 3c).

It has been demonstrated that the major birch allergens are cytoplasmic proteins and cannot be detected on the surface of dry pollen[5], while they are quickly exposed during hydration[6]. Pollen hydration occurs not only during pollination, but also in contact with body fluids and during rainfalls. Allergen elution during pollen contact with the mucosa of the upper respiratory tract is responsible for allergic rhinoconjunctivitis. However, because of their size, pollen grains are unlikely to intrude into the deeper airways, thus are unlikely to explain the occurrence of allergic asthma during or after the pollination period, especially after rainfall. The latter phenomenon can be explained by rainwater-induced abortive pollen germination connected with the release of allergen-bearing submicronic particles able to penetrate the deep airways[6, 19, 21]. Moreover, such liberated allergens may be associated with other submicronic particles (such as diesel exhaust, dust, fuel combustion) playing the role of adjuvants in allergic sensitization[3, 15, 23].

The blotting procedure applied to the tape from the Lanzoni trap causes extensive pollen grain hydration, which may induce a massive allergen release. Therefore the fact that the birch allergen abundance paralleled the presence of pollen grains (as on Fig. 3a) is not surprising. It might be even enhanced by the presence of airborne allergens released from the grains earlier due to the abortive germination mentioned above[6]. The existence of airborne allergens, connected with other submicronic particles originating from the pollen itself or from various airborne pollutants, may explain the results in Fig. 3b. Such a possibility will be elucidated further during future experiments.

The intriguing absence of allergens during the period of a detectable number of birch pollen grains (as exemplified on Fig. 3c) may be tentatively explained by reduced allergen liberation due to a temporary high concentration of some air pollutants, such as SO_2 [2]. We cannot exclude, however, that the grains were not fully mature before the pollination season and were thus faulty and non-allergenic. On the other hand, some dead, emptied grains might be trapped after the pollination season. The existence of only grains or only allergens detected during the experi-

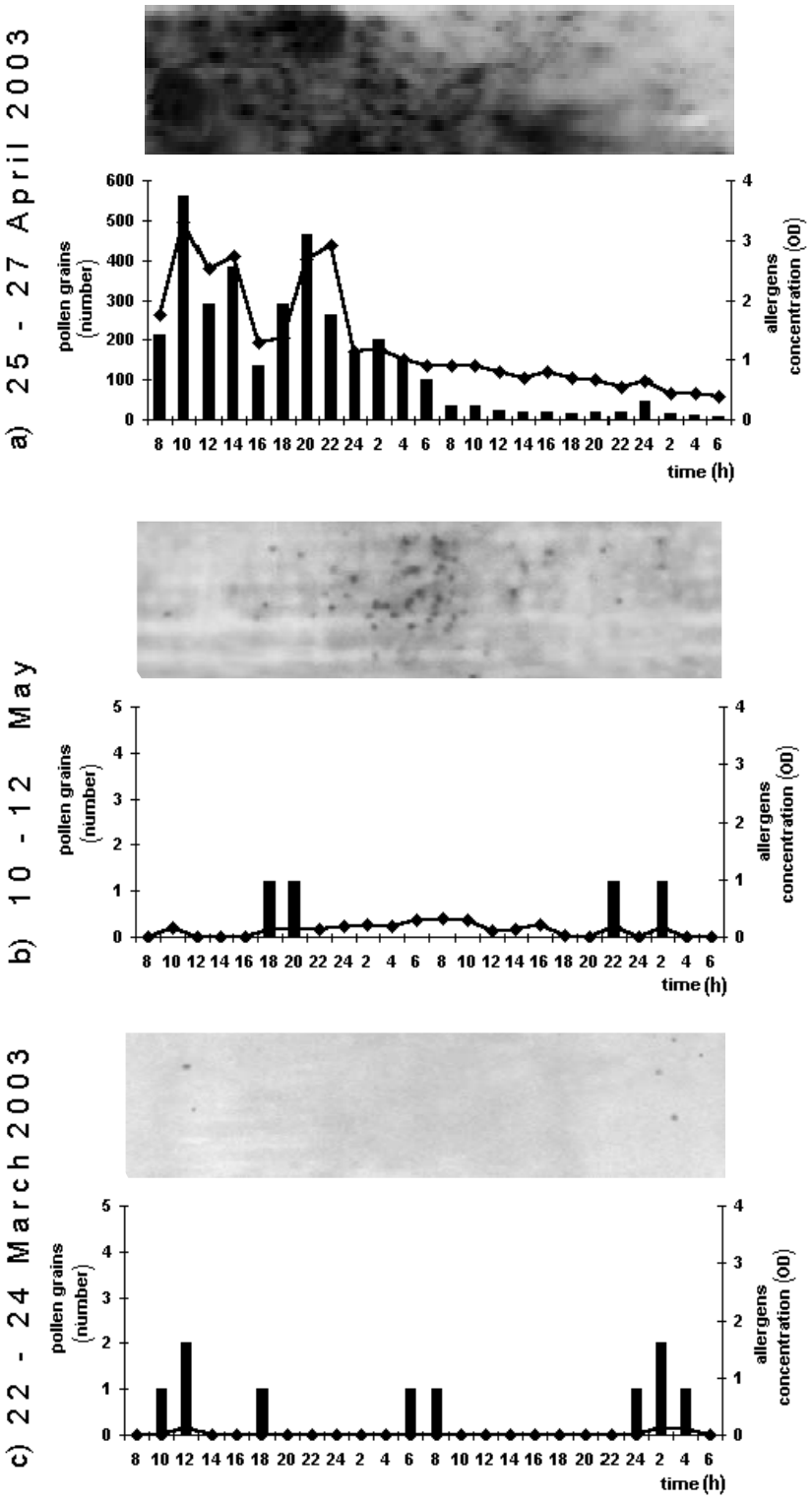

Figure 3. Analysis of *Betula* sp. pollen grains and allergens within (**a**), after (**b**) and before (**c**) the local pollination season in Krakow. Top rows: allergen visualization on the X-ray films; bottom rows: quantitative analysis of allergen concentrations (right axes, lines) and pollen grain numbers (left axes, solid bars). Compare Fig. 2.

ments presented here could also be due to sampling variability on the two different air samplers.

Further knowledge of pollination, pollen allergen release, its concentration in the air, and factors that might have impact on it is very important for clinicians and allergic persons. The results presented here confirm the notion that the traditional and commonly used pollen grain counts do not always reflect the concentrations of pollen allergens in the air. The visualization and quantification of birch pollen allergens should be further improved before being routinely performed either directly on air-sampling filters[1, 9, 10] or on blotting membranes as presented here and in other papers[4, 25].

ACKNOWLEDGMENT

We wish to thank Järgen Larsen, Gitte Nordskov Hansen and Michael Spangfort from ALK-Abelló (Hørsholm, Denmark) for providing antibodies and *Betula verrucosa* Ehrh. pollen extract. The authors are grateful to Prof. Istvan Berczi for helpful suggestions and valuable discussion.

REFERENCES

1. Acevedo F., Vesterberg O. and Bayard C. (1998): Visualization and quatification of birch-pollen allergens directly on air-sampling filtres. Allergy, 53, 594–601.
2. Behrendt H. and Becker W. M. (2001): Localization, release and bioavailability of pollen allergens: the influence of environmental factors. Curr. Opin. Immunol., 13, 709–715.
3. Diaz-Sanchez A., Dotson A.R., Takenaka H. and Saxon D. (1994): Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. J. Clin. Invest., 94, 1417–1425.
4. Ekebom A., Vesterberg O. and Hjelmroos M. (1996): Detection and quantification of airborne birch pollen allergens on PFDV membranes by immunoblotting and chemiluminescence. Grana, 35, 113–118.
5. Grote M. (1999): *In situ* localization of pollen allergens by immunogold electron microscopy: allergens at unexpected sites. Int. Arch. Allergy Immunol., 118, 1–6.
6. Grote M., Valenta R. and Reichelt R. (2003): Abortive pollen germination: a mechanism of allergen release in birch, alder, and hazel revealed by immunogold electron microscopy. J. Allergy Clin. Immunol., 111, 1017–1023.
7. Grote M., Vrtala S., Niederberger V., Wiermann R., Valenta R. and Reichelt R. (2001): Release of allergen-bearing cytoplasm from hydrated pollen: A mechanism common to a variety of grass (Poaceae) species revealed by electron microscopy. J. Allergy Clin. Immunol., 108, 109–115.
8. Hirst J. M. (1952): An automatic volumetric spore trap. Ann. Appl. Biol., 39, 257–265.
9. Holmquist L. and Vesterberg O. (1999): Luminescence immunoassay of pollen allergens on air sampling polytetrafluoroethlene filtres. J. Biochem. Biophys. Methods, 41, 49–60.
10. Holmquist L. and Vesterberg O. (2003): Immunochromatographic direct on sampling filter test for aeroallergens. J. Biochem. Biophys. Methods, 57, 183–190.
11. Jarolim E., Rumpold H., Endler A.T., Ebner H., Breitenbach M., Scheiner O. and Kraft D. (1989): IgE and IgG antibodies of patients with allergy to birch as tools to define the allergen profile of *Betula verrucosa*. Allergy, 44, 385–395.
12. Karamloo F., Schmitz N., Scheurer S., Foetisch K., Hoffmann A., Haustein D. and Vieths S. (1999): Molecular cloning and characterization of birch pollen minor allergen, Bet v 5, belonging to a family of isoflavone reductase-related proteins. J. Allergy Clin. Immunol., 104, 991–999.
13. Moore P. D., Webb J. A. and Collinson M. E. (1991). Pollen analysis. 2nd edition. Blackwell Scientific Publication, London.
14. Moverare R., Westritschnig K., Svensson M., Hayek B., Bende M., Paul G., Sorva R., Haahtela T., Valenta R. and Elfman L. (2002): Different IgE reactivity profiles in birch pollen-sensitive patients from European populations revealed by recombinant allergens: an imprint of local sensitization. Int. Arch. Allergy Immunol., 128, 325–335.
15. Nel A. E., Diaz-Sanchez D., Ng D., Hiura T. and Saxon A. (1998): Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. J. Allergy Clin. Immunol., 104, 539–554.
16. Nilsson S. and Persson S. (1981): Tree pollen spectra in the Stockholm region (Sweden), 1973–1980. Grana, 20, 179–182.
17. Pehkonen E. and Rantio-Lehtimäki A. (1994): Variations in airborne pollen antigenic particles caused by meteorologic factors. Allergy, 49, 472–477.
18. Ring J., Krämer U., Schäfer T. and Behrendt H. (2001): Why are allergies increasing? Curr. Opin Immunol., 13, 701–708.
19. Schäppi G. F., Taylor P. E., Staff I. A., Rollard J. M. and Suphioglu C. (1999): Immunologic significance of respirable atmospheric starch granules containing major birch allergen Bet v 1. Allergy, 54, 478–483.
20. Smit J., Folkerts G. and Nijkamp F. P. (2004): *Ramp*-ing up allergies: *Nramp1 (Slc 11a1)*, macrophages and the hygiene hypothesis. Trends Immunol., 25, 342–347.
21. Suphioglu C., Singh M. B., Taylor P., Bellomo R., Holmes P., Puy R. and Knox R. B. (1992): Mechanisms of grass pollen induced asthma. Lancet, 339, 569–572.
22. Szczepanek K. (1994): Pollen calendar for Cracow (southern Poland), 1982–1991. Aerobiologia, 10, 65–70.
23. Takenaka H., Zhang K., Diaz-Sanchez A., Tsien A. and Saxon A. (1995): Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: direct effects on B-cell IgE production. J. Allergy Clin. Immunol., 95, 103–115.
24. Valenta R., Duchene M., Vrtala S., Birkner T., Ebner C., Hirschwehr R., Breitenbach M., Rumpold H., Scheiner O. and Kraft D. (1991): Recombinant allergens for immunoblot diagnosis of tree-pollen allergy. J. Allergy Clin. Immunol., 88, 889–894.
25. Vesterberg O. (1995): A new luminometer for sensitive quantification by chemiluminescence of specific proteins in microtiter plates and on blot membranes. J. Biochem. Biophys. Methods., 30, 301–314.
26. Vik H., Florvaag E. and Elsayed S. (1991): Pollen Allergens. In: D'Amato G., Spieksma F. T. M. and Bonini S. (eds.): Allergenic Pollen and Pollenosis in Europe. Blackwell Scientific Publications, London, 36–44.
27. Yli-Panula E. and Ahlholm J. (1998): Prolonged antigenic activity of birch and grass pollen in experimental conditions. Grana, 37, 180–184.
28. Zając A. and Zając M. (eds.) (2001): Distribution atlas of vascular plants in Poland. Laboratory of Computer Chorology, Institute of Botany, Jagiellonian University, Cracow, 91–92.