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Timothy Grass (*Phleum pratense* L.) Pollen as Allergen Carriers and Initiators of an Allergic Response

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Key Words

Grass pollen allergens · Localization · Immunoelectron microscopy · Phl p 5 release · Air pollution

Abstract

Contrary to indoor allergen exposure (e.g. house dust mite), there is no reliable quantitative association between pollen exposure and symptoms of allergic diseases. Therefore we studied localization and release of major allergens from timothy grass (*Phleum pratense* L.) pollen using different methods and pollen grain sources. Localization of major allergens Phl p 5 and Phl p 1 was visualized by field emission scanning electron microscopy after anhydrous fixation and immunogold silver staining in a three-dimensional reconstruction; Phl p 5 was found in the cytoplasm and on the exine, Phl p 1 in the intine. No allergens were found inside the starch granules. Allergen liberation from pollen grains was studied in vitro under physiological conditions (30 min, 37°C) at pH 6.0, 7.4 and 9.0. Besides total protein measurements in the supernatant, major allergens were determined by immunoblot, Phl p 5 was quantitated by ELISA. There were striking differences in total protein and major allergen release between freshly collected and commercially available grass pollen grains as well as among freshly collected pollen between rural meadows and areas near high-traffic roads. There was a significantly different release of total protein being lowest in supernatants from commercially available pollen grains (rural/traffic vs. commercial, $p<0.001$), and of Phl p 5 major allergen (rural>traffic>commercial, $p<0.005$). Therefore, allergen bioavailability seems to be an important parameter in order to establish reliable dose-response relationships for the outdoor allergen response. Pollen grains incubated in aqueous protein-free buffer solution were also found to secrete significant amounts of eicosanoids namely prostaglandin E2 and leukotriene B4. Pollen grains thus do not act only as allergen carriers but also might have important implications on early events as initiators of allergy.

Introduction

The prevalence of allergic diseases has increased worldwide during the last decades [1]; the reasons for this increase are unknown, among many hypotheses [2–4] the aspect of allergen exposure as well as the influence of environmental pollutants has gained substantial scientific attention [3, 5–9].

In order to study dose-response relationships between disease outcome and exposure measurements and to moni-

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tor the effects of ambient air ingredients upon human health with special reference to allergy it is necessary to have solid information on dose-response relationships between allergen content in the outdoor air and clinical response. So far these data are missing. Contrary to indoor allergen exposure – e.g. housedust mite – there is no reliable quantitative association between pollen exposure and symptoms of allergic diseases.

Therefore we studied the localization and release of major allergens from timothy grass (*Phleum pratense* L.) pollen using different methods and pollen grain sources. In addition we studied the secretion of other possible proinflammatory substances from pollen grains and were able to demonstrate that pollen contain and release substantial amounts of eicosanoids such as prostaglandin E2 and leukotriene B4 under physiological conditions.

Materials and Methods

Pollen Grain Sources

The pollen grains studied were purchased from commercial producers (Allergon, Sweden: grass pollen, batch 011396102; pine pollen, batch 013898901; Sigma, Germany: birch pollen batch 19F0783) as well as freshly collected from pollinating *P. pratense* plants growing either on a rural meadow in upper Bavaria and from the roadside of a high-traffic road in Southern Munich.

Allergen Localization

Localization of the major allergens Phl p 1 and Phl p 5 was demonstrated by light as well as by scanning electron microscopy after anhydrous fixation of the samples. Visualization was performed by silver-enhanced immunogold staining and by fluorescence microscopy.

Allergen and Eicosanoid Liberation

To study allergen release and eicosanoid secretion from pollen grains the samples were incubated in vitro in phosphate-buffered saline (30 min at 37 °C at pH 6.0, 7.4, and 9.0). In the supernatants total protein content was measured. Two major allergens of *P. pratense*, Phl p 1 and Phl p 5, were identified using immunoblot technique. Phl p 5 as well as the major allergen of birch, Bet v 1, were quantitated by enzyme-linked immunosorbent assay [10, 11] using monoclonal antibodies, kindly provided by Prof. Fiebig (Allergopharma, Reinbek, Germany). Concentrations of the eicosanoids prostaglandin E2 and leukotriene B4 were measured using commercially available enzyme immunoassays (Amersham-Pharmacia, Germany). Eicosanoids were further identified by HPLC.

Statistical Analysis

If not mentioned otherwise mean values and standard deviations are given. Statistical significance of differences was calculated using the Student t test.

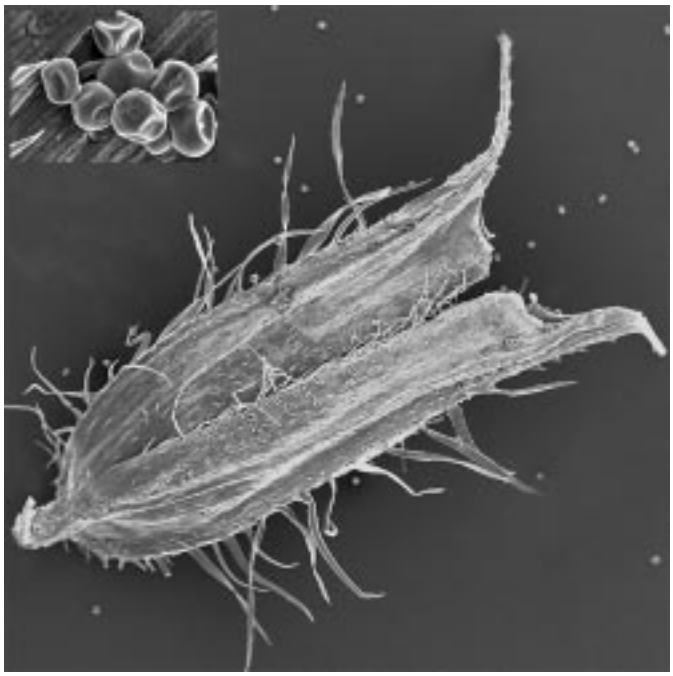

Fig. 1. Scanning electron-microscopic picture of an anther of timothy grass (*P. pratense* L.) and its pollen grains. ×20. The inset shows pollen grains on the outside. ×750.

Results

*Allergen Localization of Major Allergens from Grass Pollen (*P. pratense *L.)*

Pollen from commercial sources as well as from pollen anthers of *P. pratense* L. which had been freshly collected just prior to pollination (fig. 1) were investigated. As shown after strictly anhydrous fixation and by use of the monoclonal antibody (mAb) Bo1 major pollen allergen Phl p 5 is located in the cytoplasm of the pollen grain and on the surface of the exine. There is no significant allergen localized within or associated with the starch granules (fig. 2a, c). Phl p 1 allergen was detected by the use of mAb Ig12 and was found in outer parts of the cytoplasm but concentrated in the intine of the pollen grains (fig. 2b, d). This specific distribution pattern was confirmed by field emission scanning electron microscopy in a three-dimensional reconstruction.

Allergen Liberation from Pollen Grains

There was a pH-dependent release of total protein from birch, grass and pine pollen as well as of Bet v 1 and Phl p 5 allergen with maximal values of protein released at pH 9.0 and of allergen at pH 7.4 (table 1). About one third of total protein was Bet v 1 allergen. In contrast, Phl p 5 represents

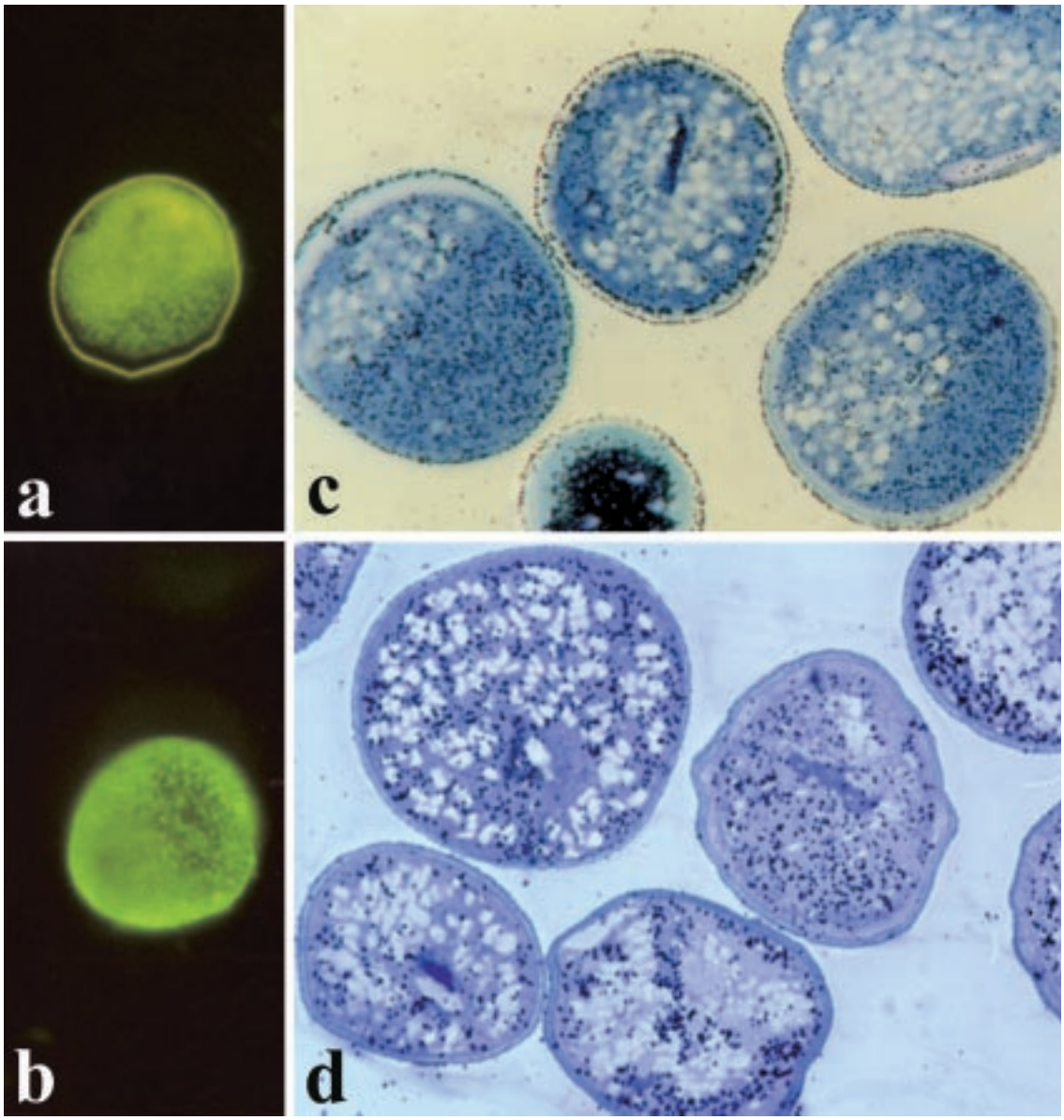

Fig. 2. Demonstration of major allergens Phl p 5 using mAb Bo1 (**a**, **c**) and Phl p 1 using mAb Ig12 (**b**, **d**) within pollen grains from *P. pratense* L. by fluorescence microscopy and after immunogold silver staining at the light microscopic level. Magnification: ×630 (**a**, **b**); ×1200 (**c**, **d**).

only <10% of total protein. The absolute amount of allergen, however, was equal in both species.

There were striking differences in total protein and major allergen release between freshly collected and commercially available grass pollen grains as well as between freshly collected pollen from rural meadows and roadside areas. There was a significantly different release of total protein (rural/traffic vs. commercial, $p<0.001$) and of Phl p 5 liberation (rural > traffic > commercial, $p<0.005$). The lowest allergen levels were found in supernatants from commercially available pollen grains: 9.2±1.4 μg Phl p 5 per 10 mg pollen compared to 35.1±4.2 μg in rural and 21.7±2.3 μg in traffic-associated freshly collected pollen (fig. 3). When we calculated the bioavailability of aeroallergens after pollen grain contact with aqueous surfaces there were striking differences both in protein and in allergen release between birch, grass and pine pollen (table 1).

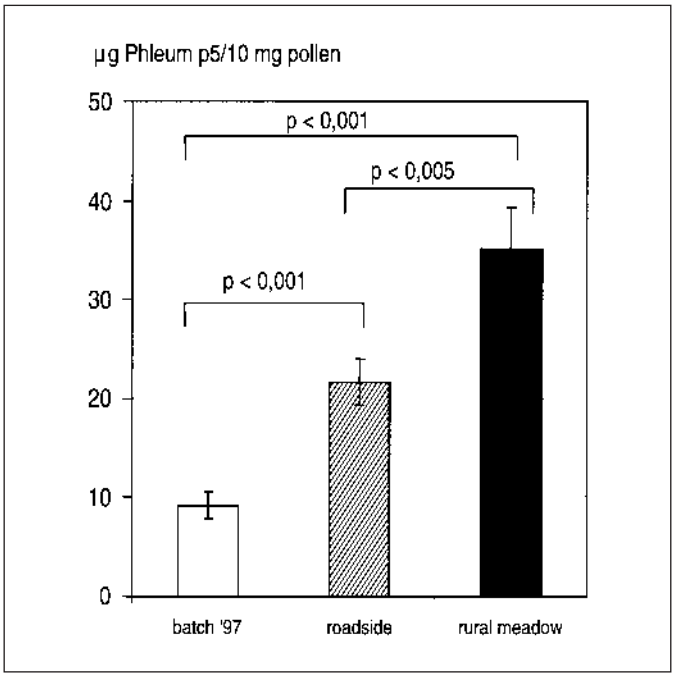

Fig. 3. Release of major allergen Phl p 5 from timothy grass pollen collected in 1997 from rural meadow or from traffic-related area compared to a commercial batch of 1997. 10 mg pollen were incubated in 1 ml PBS at pH 7.4 over 30 min at 37°C. Means are given ± SD (n = 8).

Pollen Grains as Initiators of an Allergic Response by Releasing Eicosanoids

Furthermore pollen grains incubated in phosphate-buffered saline were found to secrete significant amounts of eicosanoids, namely prostaglandin E2 and leukotriene B4 (table 2). Prostaglandin E2 content was highest in birch pollen supernatants. Substantial amounts of leukotriene B4 could be detected to be released from birch and grass pollen, but not from pine pollen.

Discussion

Pollen grains are the major source of outdoor aeroallergens [12]. Allergen exposure is the primary condition for predisposed individuals to develop an allergic sensitization as well as for elicitation of symptoms of allergic inflammation in sensitized individuals. Many allergists still believe that the allergic response starts with the contact of the allergen on the surface of the antigen-presenting cell in the nose or the lung. As a matter of fact, most studies dealing with the allergic reaction use allergen extracts as stimulus. Under natural exposure conditions, however, the bioavailability of allergens depends on the allergen liberation from internal binding sites within the allergen carrier, namely the pollen grain [13]. This process of activation of the pollen grain results in the release of allergens and occurs under humid conditions either at the mucosal surface or maybe already in the ambient air [11, 14–16].

The results of our study show clearly that major grass pollen allergens are primarily localized within the pollen grain in specific patterns. There was no major pollen allergen found inside the starch granules. During activation allergens seem to be transported through microchannels in the exine onto the surface in order to be liberated. This process of allergen release is not associated with pollen disruption nor is it connected with tube formation.

We found striking differences both in protein and allergen release from pollen grains of different sources and species. So for example the percentage amount of allergen

Table 1. Bioavailability of aeroallergens in vitro

Pollen	pH	Total protein µg/ml	Allergen µg/ml	Allergen % total protein
Birch	4.0	20.9	7.1	33.9
	6.0	21.6	6.2	28.8
	7.4	30.0	9.3	31.3
	9.0	32.6	8.7	26.7
Grass	6.0	132.0	8.0	6.0
	7.4	167.1	9.2	5.5
	9.0	171.0	8.3	4.9
Pine	6.0	3.7		
	7.4	8.9		
	9.0	13.1		

10 mg pollen/ml PBS, 30 min, 37°C.

Table 2. In vitro release of prostaglandin E2 and leukotriene B4 from birch (*Betula alba* L.), grass (*P. pratense* L.), and pine (*P. silvestris* L.) pollen

	Pollen, pg/ml		
	birch	grass	pine
Prostaglandin E2[a]	2,599±93	696±15	625±59
Leukotriene B4[b]	348±19	391±19	30±1

10 mg pollen/ml PBS, pH 7.4, 37°C (mean ± SD, n = 8).
[a] (grass vs. pine: nonsignificant; birch vs. grass/pine: $p < 0.001$.
[b] Birch vs. grass: nonsignificant; birch/grass vs. pine: $p < 0.05$.

released from birch pollen is much higher as that from grass pollen on the basis of total protein release. The lowest amount of protein released into aqueous solution was found for *Pinus silvestris* pollen. This raises the question as to whether allergenic 'potency' of various allergens has to be redefined regarding the different steps of allergen release prior to contact with the host's immune system. We also found striking differences in allergen release between pollen collected from rural meadows not exposed to automobile emissions and pollen collected along the roadside of a high-traffic road. It is too early to speculate about the possible implications of these findings for the role of environmental pollutants in the induction or elicitation of allergic reactions [5–9, 17].

The most exciting finding of the study was the fact that the pollen grain itself contains and liberates proinflammatory mediators known to play an important role in allergic reactions namely eicosanoids. To our knowledge this is the first report of this finding. In our preliminary studies we found substantial amounts of prostaglandin E2 and leukotriene B4 in aqueous supernatants of pollen grains from timothy grass. This finding opens a new dimension of understanding the early events in allergic sensitization. It indicates that the process does not start with the contact of allergen on the surface of a macrophage or antigen-presenting cell, but begins much earlier. Allergen carriers (e.g. pollen grains) first have to release their allergens in the humid environment of an appropriate carrier (i.e. mucosal surface, skin, ambient air). At the same time pollen release eicosanoids with well-known chemoattractant or immunomodulatory properties which can attract and activate inflammatory cells present in the upper part of mucous membranes. We hypothesize that a process of 'initiation of allergy' precedes allergen/antigen-presenting cell interaction and may be the very first step in the process of atopic sensitization which then includes antigen presentation, T cell activation, antibody production and development of immunological memory. Different allergen release patterns regarding different pollen sources and species might contribute to the well-known variability of the response to pollen exposure in allergic individuals. The aspect of allergen bioavailability has to be considered more thoroughly in the future since it seems to be an important factor in establishing reliable dose-response relationships for outdoor allergen exposure.

Acknowledgement

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