

Allergens are transported through the respiratory epithelium

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We used a top-down approach with a wide repertoire of wet laboratory and *in silico* techniques for analyzing the pathogenesis of early events within the type I allergic reactions. We could show a caveolar-dependent transport of the birch pollen allergen through the respiratory epithelium of allergic patients but not of their healthy controls. The application of discovery-driven methodologies can provide new hypotheses worth further analyses of complex multifactorial diseases such as type I allergy.

KEYWORDS: allergy • caveolae • epithelium • pollen allergen

Background & study plan

Allergic conjunctivitis and rhinitis are type I hypersensitivity reactions caused by antigens, such as pollen allergens, cross-linking specific anti-IgE molecules on the previously presensitized mast cells. During the past years, a great majority of work related to the pathogenesis of pollen allergy has focused on the mechanisms of the immune responses elicited by the allergen [1–5]. We have initiated a large-scale effort to analyze the role of the respiratory epithelium in connection with allergic reactions. We have previously developed a noninvasive application of confocal light microscopy enabling direct and repeatable quantitative analyses of the very early phases of conjunctival allergic inflammation in human patients [6,7]. A challenge with the birch pollen allergen Bet v 1 led, within minutes, to leukocyte extravasation into the conjunctival sites of inflammation in allergic individuals, but not in healthy individuals. Thus, the major question was: what takes place at the epithelial surfaces within the first minutes after contact with pollen allergens?

Allergic symptoms cause much morbidity and thus are one major indirect reason for losses of productivity in society, as up to 15–20% of the European population suffer from pollen allergies [8]. Type I allergy is an example of an acute disease, where its causative agents, such as the birch pollen allergen Bet v 1, have been identified. Conjunctival, nasal, and other respiratory epithelial cells and tissues can be obtained from healthy individuals and allergic

patients. To minimize the *in vitro* artefacts, we collected, analyzed and compared only freshly isolated epithelial cell swabs and epithelial tissue biopsies from healthy control subjects and birch pollen-allergic patients. These specimens were analyzed before and after the *in vivo* birch pollen challenge and the following data domains were gathered: transcriptomics with various platforms, Bet v 1-associated proteins with various high-performance liquid chromatography (HPLC) methods combined with tandem mass spectrometry (MS/MS) analysis, and light and electron microscopy analyses [9–12].

This is the first part of a study of a human disease at the systems level. We identified a group of birch-allergic subjects and verified their clinical symptoms during the birch pollen season with a positive skin prick test. An age-matched group of healthy controls with no symptoms and a negative skin prick test was also identified. We then collected conjunctival and nasal epithelial cell swabs and biopsies from all subjects during the winter when there was no birch pollen in the air, and thus even the allergic patients did not present any symptoms. During this previously nonsymptomatic period, we could also apply *in vivo* perturbation by an application of pollen allergen to the conjunctiva, as well as under the inferior meatus of the nasal cavity, which caused rapid symptoms in allergic subjects, but no manifestation in healthy controls. We collected a new set of cell and tissue specimens, and analyzed all specimens for further analyses.

Birch pollen allergen, Bet v 1, binds to & is transported through the respiratory epithelium

First, we began to analyze contact between the Bet v 1 allergen and the respiratory epithelium. *In vivo* challenge with the birch pollen allergen led to a very rapid binding of the major allergen Bet v 1 to both the stratified conjunctival as well as pseudostratified nasal epithelium. Already after 1 min, Bet v 1 was localized in clusters on the epithelial surfaces in allergic patients, but not in healthy control subjects. Within minutes, the Bet v 1 allergen was also taken up into the epithelial cells of allergic patients. This uptake process was shown to be an active lipid raft and caveolar-dependent process. A great majority of Bet v 1 allergen within the epithelial cells was also colocalized with caveolar markers, such as caveolin 2 or flotillin 2. Analyses with immunoelectron microscopy, using some 500 merged frames taken at 27,000 $\times$ magnification, revealed that the Bet v 1 allergen could traffic through the whole nasal epithelium within a few minutes and localize in the mast cells lying under the basement membrane [9,11]. Thus, the first line of evidence gathered by various microscopy techniques suggested that the Bet v 1 allergen binds to and trafficks through the respiratory epithelium via a lipid raft and caveolar-dependent process.

To add further proof to this novel hypothesis of active allergen transport through the epithelium, we next used affinity columns, HPLC and mass spectrometry. We wanted to identify the Bet v 1-associated proteins acting as putative receptors and/or participating in intracellular transport through the epithelium. We performed affinity chromatography with the covalently matrix-bound tandem affinity purification (TAP)-tagged Bet v 1 and nasal epithelial cell lysates taken during the winter season from both healthy and allergic subjects. The Bet v 1-associated proteins were eluted from the column and subjected to 2D HPLC and MS/MS stop-flow analyses. The peptides were identified with database-related and *de novo* identification, and a shortlist of Bet v 1-associated proteins was generated with very stringent criteria. Among the 16 proteins found on this shortlist to associate with Bet v 1 in allergic but not in healthy epithelial cells were six proteins (ACTG, PLEC1, STML2, KCNA5, CALM and ANXA2), previously characterized to be located within caveolae. Thus, the relative proportion of caveolar proteins within the Bet v 1-associated list of proteins was high – 38% as compared with 1% of caveolar proteins within the human proteome. Concomitantly, no caveolar proteins were found to associate with Bet v 1 in the epithelial cell lysates from healthy controls [9]. This observation on its own clearly supported the hypothesis that the Bet v 1 allergen could travel through the epithelial tissues within caveolae.

The third independent data domain we began to work with was 3D modeling of Bet v 1 and its binding ligands. Previous literature had suggested that Bet v 1 allergen could bind to cholesterol [13]. As the birch pollen allergen Bet v 1 enters the epithelium of allergic patients via the caveola, known to be enriched with cholesterol and glycolipids, we began to analyze its putative amphiphilic and lipid ligands on the atomic level

using molecular modeling and computational ligand docking. We carried out a 3D-modeling docking with both experimentally verified Bet v 1 ligands as well as larger lipid molecules for which no experimental affinity data were available. Our results suggested that the hydrophobic cavity of Bet v 1 has different binding sites for different ligands and we could identify the groups of ligand-specific amino acids. Bet v 1 proteins may also be able to bind and transport more complex amphiphilic molecules such as ceramides and sphingomyelins, which are enriched on caveolar surfaces. We suggested a binding mode, where the hydrophobic tail groups of lipids are located in the Y-shaped cavity within the Bet v 1 molecule, while the polar head group remained solvent accessible [14]. This would allow Bet v 1 to bind glycolipids, such as gangliosides, which are abundant on caveolae. Thus, our 3D modeling *in silico* work suggests that Bet v 1 binds to caveolar amphiphilic and lipid ligands. This could provide a molecular mechanism for pollen entry into the epithelial tissue of allergic patients.

Allergy is a disease of the epithelium

The fourth independent data domain we collected and analyzed was transcriptomics. We collected nasal epithelial cell swab specimens from both birch pollen allergic patients and healthy controls during the nonsymptomatic winter season and during the symptomatic spring season at weekly intervals, starting at the onset of the birch pollen season. At the beginning of the birch pollen season in southern Finland (which is in late April), the patients but not the controls began to show clinical symptoms. During the course of this experiment, the healthy controls remained asymptomatic while the allergic subjects gradually generated more and more severe symptoms [10].

First of all, the epithelial transcriptome had over 100 differentially expressed genes during the asymptomatic winter period. This would suggest that the state of the epithelium is already different between patients and controls, even though nobody presented with any symptoms. The time-series analyses provided further interesting data. The epithelium sampled from healthy subjects displayed some 40 significantly altered transcripts during the birch pollen season, out of which some 20 were unique for this group and the rest were also seen to be modified in the allergic patients. Of these uniquely regulated genes, a significantly high proportion (40%) belonged to the Gene Ontology category immune response (GO:0006955) [101]. The members of the immune response modified in healthy subjects were: CXL10, CXCL6, CXCL9, CXCR2, IL-1 and IL-8, NKG7, PGSG and FPR1. This immune response category has 550 proteins in total and represents only a small percentage of all annotated proteins within the human proteome [101]. Thus, we could show that the pollen allergen induced a strong epithelial immune response in the clinically nonreactive healthy subjects while this same response was not detected in the allergic subjects [10].

On the other hand, the allergic subjects showed a set of some 100 uniquely regulated transcripts (converted for further analyses to proteins) in nasal epithelial specimens during the progression of

their clinical symptoms. A detailed analysis showed that dyneins are among the most enriched gene ontology categories. The whole human proteome has only 19 dynein proteins (0.1%), while we observed six of them among the 100 modified epithelial proteins (5.7%) in the allergic epithelium. Dyneins are cellular molecular motors responsible for both epithelial ciliary movements as well as intracellular caveolar cargo traffic along microtubuli [10].

Network analyses [102] of these 100 allergic-specific genes/proteins, displaying altered expression levels during the time series analyses, indicated that the annotated molecular functions of these proteins are often linked to the actin cytoskeleton and caveolar proteins such as aquaporin 5.

In addition, other groups have shown the altered cellular state of the epithelial cells in allergic patients versus healthy controls. The hyporesponsiveness of the allergic epithelial cells was observed in *in vitro* cultivations after allergen perturbation [15–17]. Likewise, allergen traffic into the respiratory epithelial cells has also recently been shown with the timothy grass pollen major allergen, Phl p 1 [18,19].

Expert commentary & five-year view

In this article, we summarize the results obtained from conjunctival epithelial cells. Comparing the conjunctival epithelial cells and tissues from healthy and allergic subjects, we have shown with light and electron microscopic analyses that the Bet v 1 birch pollen allergen binds to epithelial cells within minutes, even during the nonsymptomatic winter season, but only in allergic individuals. Bet v 1 was also transported through the morphologically intact epithelium and reached mast cells located close to or under the basal lamina during the nonsymptomatic winter season in allergic patients. Colocalization together with caveolar markers and affinity experiments with mass spectrometry suggested that this traffic was dependent on caveolae (FIGURE 1). Finally, transcriptomics provided evidence that the respiratory epithelium also differs between healthy controls and allergic patients during the nonsymptomatic winter season and, furthermore, that the unique sets of genes alter in both groups in response to the Bet v 1 birch pollen challenge.

There are several novel aspects to be analyzed. Our preliminary evidence indicates that grass pollen allergens are not taken up by the epithelium of birch pollen-allergic individuals. How other allergens interact with the respiratory epithelium needs

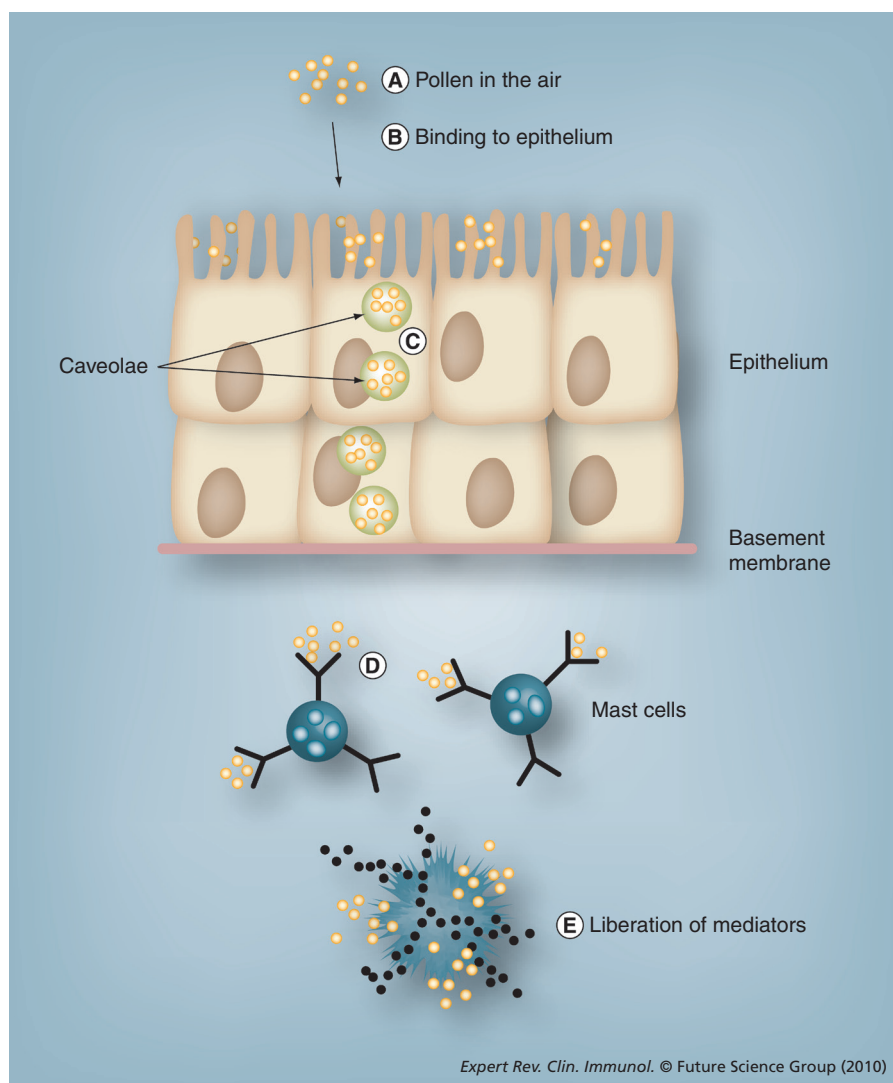


Figure 1. Allergens meet the respiratory epithelium. (A) Birch pollen is in the air during the allergy season. (B) Bet v 1, the major allergen in birch pollen, binds in clusters to the surface of respiratory epithelial cells in allergic patients, but not in healthy subjects. (C) Bet v 1 allergen is actively transported through the epithelium within the caveolae. (D) Bet v 1 allergen passes through the basement membrane and makes contact with specific anti-Bet v 1 IgE molecules bound with the corresponding FcεR1 receptors to mast cells. (E) This interaction induces mast cells to liberate their mediators, such as histamine, causing the clinical symptoms of acute allergic reactions.

to be further studied. Whether the active role of lipid rafts and caveolae are a cause or a consequence of the observations described in this article must also be addressed. How do allergens pass the respiratory tract the first time in not-yet allergic individuals who are about to become allergic? Many of these and other issues are currently under further investigations.

Thus, taken together, the application of discovery-driven methodologies on human conjunctival and nasal epithelial cells and tissues can provide new hypotheses worth further analyses on the molecular mechanisms of a complex multifactorial disease, such as type I birch pollen allergy. Learning novel, unpredictable aspects from complex immunological diseases can provide future targets for drug development.

Financial & competing interests disclosure

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Key issues

- Bet v 1 birch pollen allergen binds to epithelial cells and tissues within minutes, even during the nonsymptomatic winter season, in allergic individuals.
- Bet v 1 never binds to, nor is transported through, the epithelium of healthy individuals.
- Bet v 1 is transported through the epithelium to mast cells within minutes in allergic patients.
- Microscopic affinity experiments with mass spectrometry and transcriptomics all suggested that Bet v 1 is transported via the caveolae.
- Transcriptomics indicate that the respiratory epithelium differs between healthy controls and allergic patients, during the nonsymptomatic winter season.
- The application of discovery-driven methodologies can provide new hypotheses worth the further analyses of complex multifactorial diseases, such as type I allergy.

References

Papers of special note have been highlighted as:

- of interest

- 1 Averbeck M, Gebhardt C, Emmrich F, Treudler R, Simon JC. Immunologic principles of allergic disease. *J. Dtsch Dermatol. Ges.* 5(11), 1015–1028 (2007).
- 2 Bohle B. The impact of pollen-related food allergens on pollen allergy. *Allergy* 62(1), 3–10 (2007).
- 3 D'Amato G, Cecchi L, Bonini S *et al.* Allergenic pollen and pollen allergy in Europe. *Allergy* 62(9), 976–990 (2007).
- 4 Fukushima A. Roles of T-cells in the development of allergic conjunctival diseases. *Cornea* 26(9 Suppl. 1), S36–S40 (2007).
- 5 Larche M. Regulatory T cells in allergy and asthma. *Chest* 132(3), 1007–1014 (2007).
- 6 Helinto M, Renkonen R, Tervo T *et al.* Direct *in vivo* monitoring of acute allergic reactions in human conjunctiva. *J. Immunol.* 172(5), 3235–3242 (2004).
- 7 Kirveskari J, Vesaluoma MH, Moilanen JA *et al.* A novel non-invasive, *in vivo* technique for the quantification of leukocyte rolling and extravasation at sites of inflammation in human patients. *Nat. Med.* 7(3), 376–379 (2001).
- 8 Haahela T, von Hertzen L, Makela M, Hannuksela M. Finnish Allergy Programme 2008–2018 – time to act and change the course. *Allergy* 63(6), 634–645 (2008).
- Outlines action points needed to approach allergic diseases from a new angle.
- 9 Joenväärä S, Mattila P, Renkonen J *et al.* Caveolar traffic through nasal epithelium of birch pollen allergen Bet v 1 in allergic patients. *J. Allergy Clin. Immunol.* 124, 135–142 (2009).
- Outlines the Bet v 1 transport mechanisms through the nasal epithelium.
- 10 Mattila P, Renkonen J, Toppila-Salmi S *et al.* Time-series nasal epithelial transcriptomics during natural pollen exposure in healthy subjects and allergic patients. *Allergy* (2009) (Epub ahead of print).
- 11 Renkonen J, Mattila P, Lehti S *et al.* Birch pollen allergen Bet v 1 bind to and travels through conjunctival epithelium in allergic patients. *Allergy* 64, 868–875 (2009).
- Outlines the Bet v 1 transport mechanisms through the conjunctival epithelium.
- 12 Renkonen J, Mattila P, Parviainen V, Joenväärä S, Toppila-Salmi S, Renkonen R. A network analysis of the single nucleotide polymorphisms in acute allergic diseases. *Allergy* (2009) (Epub ahead of print).
- 13 Mogensen J, Wimmer R, Larsen J, Spangfort M, Otzen D. The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.* 277, 23684–23692 (2002).
- 14 Mattila K, Renkonen R. Modeling of Bet v 1 binding to lipids. *Scand. J. Immunol.* 70(2), 116–124 (2009).
- 15 Vroiling AB, Duinsbergen D, Fokkens WJ, van Drunen CM. Allergen induced gene expression of airway epithelial cells shows a possible role for TNF- α . *Allergy* 62(11), 1310–1319 (2007).
- 16 Vroiling AB, Fokkens WJ, van Drunen CM. How epithelial cells detect danger: aiding the immune response. *Allergy* 63(9), 1110–1123 (2008).
- 17 Vroiling AB, Jonker MJ, Luiten S, Breit TM, Fokkens WJ, van Drunen CM. Primary nasal epithelium exposed to house dust mite extract shows activated expression in allergic individuals. *Am. J. Respir. Cell. Mol. Biol.* 38(3), 293–299 (2008).
- 18 Blume C, Foerster S, Gilles S *et al.* Human epithelial cells of the respiratory tract and the skin differentially internalize grass pollen allergens. *J. Invest. Dermatol.* 129(8), 1935–1944 (2009).
- 19 Röschmann K, Farhat K, König P, Suck R, Ulmer A, Petersen A. Timothy grass pollen major allergen Phl p 1 activates respiratory epithelial cells by a non-protease mechanism. *Clin. Exp. Allergy* 39(9), 1358–1369 (2009).

Websites

- 101 The Gene Ontology
<http://amigo.geneontology.org/cgi-bin/amigo/go.cgi>
- 102 Mediceal
www.mediceal.com

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