Chapter 2
The multi-facettted role of allergen exposure to the local airway mucosa

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Allergy. 2013 Feb;68(2):152-60
SUMMARY

Airway epithelial cells are the first to encounter aeroallergens and therefore have recently become an interesting target of many studies investigating their involvement in modulation of allergic inflammatory responses. Disruption of a passive structural barrier composed of epithelial cells by intrinsic proteolytic activity of allergens may facilitate allergen penetration into local tissues and additionally affect chronic and ongoing inflammatory processes in respiratory tissues. Furthermore, the ability of rhinoviruses to disrupt and interfere with epithelial tight junctions may alter the barrier integrity and enable a passive passage of inhaled allergens through the airway epithelium. On the other hand, epithelial cells are no longer considered to act only as a physical barrier towards inhaled allergens, but also to actively contribute to airway inflammation by detecting and responding to environmental factors. Epithelial cells can produce mediators, which may affect the recruitment and activation of more specialized immune cells to the local tissue and also create a micro-environment in which these activated immune cells may function and propagate the inflammatory processes. This review presents the dual role of epithelium acting as a passive and active barrier when encountering an inhaled allergen and how this double role contributes to the start of local immune responses.

BACKGROUND

The approach to understanding allergy seems to be changing from a reductionistic view to a more holistic view. This comes with the realization that the cells of the immune system fulfill their roles in the defense against potential threats to the organism in the context of a local tissue that is exposed to the environment. This applies both for the beginning of an immune response where the potential threat is first detected and at the end of an immune response where immune effector cells try to eliminate this threat.

When allergens are able to settle in our mucus, some significant changes may occur. Spores from fungi and pollen from grasses or trees may start to germinate and release their contents, and the proteins that make up the allergenic mixture may get processed by the proteases that are part of the mucus layer. All these effects may modify the allergenic capacity of allergens well before they come into contact with any cells of the immune system. If we also consider that the allergens would become mixed with other environmental factors like microbial products from resident bacteria or viruses then the triggers we study in our experimental settings are probably far removed from those we would encounter in real life. Figures 1 and 2 show a representation of the airway mucosa that will lead us through this review.

Uptake of allergens by epithelial cells/transport through the epithelium

The first questions that arise when allergens encounter the airway epithelium are i) how do they overcome the epithelial barrier to be recognized by immune cells? and ii) does the airway epithelium play a passive or active role in this context?

Assuming a rather passive position of airway epithelium, much attention has been paid to intrinsic features of allergens that would facilitate their passage through the epithelium. Proteolytic activity as a general feature of major allergens has been proposed to be involved in the pathogenesis of allergies by enabling the passage of allergens through the epithelial barrier. Airborne allergens derived from
sources such as house dust mite (HDM), cockroach, fungi, or pollen were identified as cysteine, serine or aspartic proteases (1,2). Der-p1, a major allergen from the house dust mite *Dermatophagoides pteronyssinus*, has been shown to be a cysteine protease (3). Furthermore, HDM derived Der-p3 also displays the properties of a cysteine protease, while Der-p6 and Der-p9 are functional serine proteases. Also for Pen-ch13, an allergenic component from *Penicillium chrysogenum*, a serine protease activity could be described (1). Phl-p1, a major allergen from Timothy grass pollen (*Phleum pratense*), shows a high sequence homology to the cysteine proteases papain and Der-p1 (4). While for recombinant Phl-p1 expressed in *Pichia pastoris* a proteolytic activity could been shown (5), natural Phl-p1 failed to display proteolytic abilities both in vitro and ex vivo (6).

Apart from these intrinsic features of allergens, a disruption of airway epithelium barrier function is also induced by many environmental factors or is a phenomenon in many diseases. Exposure of bronchial epithelial cells to cigarette smoke extract with concentrations comparable to those found in smokers induces the development of paracellular gaps and a decrease in transepithelial resistance.

Cigarette smoke exposure induces a more than 3-fold increase in allergen penetration and the resulting increased subepithelial allergen concentrations provoke a substantial augmentation of histamine release from sensitized basophils (7). Furthermore, an increased alveolar epithelial permeability has been found in cigarette smokers (8) and mice that are exposed to cigarette smoke become more easily sensitized to respiratory allergens (9). Thus, cigarette smoke is a potent factor of reducing the barrier function of the epithelium and may contribute to sensitization and increased allergic inflammation.

It is well established that viral respiratory tract infections are important triggers of acute exacerbations of chronic airway diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) (10-13). Furthermore, during infancy these infections are associated with an increased risk for the development of asthma later in life (14, 15). Airway epithelial cells are the principal host for respiratory viruses. Influenza virus and respiratory syncytial virus (RSV) have been shown to have cytotoxic effects on bronchial epithelium (16), furthermore, respiratory viruses can interfere with the structural integrity of tight junctions, leading to an increase in paracellular permeability. Rhinoviruses are able to disrupt tight junctions (17) as well, and infection of AEC with RSV results in a decreased transepithelial resistance (18). In addition, mediators produced by AEC or infiltrating immune competent cells upon viral infection may influence cell-to-cell contact by interacting with tight junctions and desmosomes. This disruption of the barrier integrity will last even post infection so may again enable a passive passage of inhaled allergens through the airway epithelium (19).

In some diseases the epithelial integrity is affected, which would also allow allergens to penetrate our defenses more readily. In this line, recent publications show that loss-of-function mutations within the filaggrin gene are linked to atopic dermatitis (AD), with AD patients having an increased frequency of filaggrin null mutations (20, 21). As filaggrin, among other processes, contributes to the ultrastructural organization of intermediate filaments (22), dysfunctional filaggrin has been suspected to be responsible for the impaired barrier function of the skin, enabling allergen penetration followed by local inflammation. Atopic dermatitis is just one example, as epithelial disruptions are also seen in asthma (23), chronic rhinosinusitis with nasal polyposis (24), and when we extend our view to the digestive tract, also in inflammatory bowel disease (25) and Crohn’s disease (26). These disruptions are
likely to be the reason for the increased allergen penetration seen in quite some studies (27-29). An excessive mucosal permeability has been reported to exist in atopic individuals (30). However, in a more recent study involving patients with perennial HDM-induced allergic rhinitis, the absorption capacity of the airway mucosa did not differ from that recorded in healthy subjects (31). Others studies showed a rapid and high absorption of Ovalbumin through the respiratory epithelium in both the nose and lower airways, with a higher absorption in the lower airways than in nasal mucosa (32).

Figure 1. Cross-sectional overview of the airway mucosa tracing the different paths an allergen may travel. In panel A we see the action of allergen on the proteins of the tight junctions (ZO, Occludin, Claudin) or their contribution to TLR-4 signaling. In panel B the two routes allergen may follow after epithelial uptake, leading either to their transport to the subepithelial layer or toward the MHC compartment of the epithelial cells, and in addition, the way protease components of allergen may activate epithelial cells through protease activated receptors (PAR). In the central part we see the allergen reaching professional antigen presenting cells that with the help of epithelial mediators may induce a Th2 response.

Several lines of evidence suggest that intrinsic proteolytic activity of allergens may indeed facilitate allergen penetration into local tissues, but on the other hand the proteolytic activity may also affect chronic inflammation in a more direct fashion. In this context proteolytic allergens have been shown to down-regulate the antiprotease-based defense within the mucosa, leading to tissue damage and immune activation, with group 1 mite allergens degrading airway α1-antitrypsin inhibitor (33), degrading and inactivating lung surfactant proteins A and D (34), and
cleaving elastase-specific inhibitors and secretory leukocyte protease inhibitors (35, 36). The importance of the balance or conversely misbalance between protease and their inhibitor could also underlie the pathogenic mechanism in Netherton's syndrome. This disease with severe disruptions in the skin integrity has been linked to mutations in SPINK5, a gene encoding a protease inhibitor (37).

In contrast to this rather passive role of the epithelium, recently there is an increased interest in the process of direct uptake of allergens by AEC, putting these cells in a more active role (38, 39). Blume et al. have been able to show that there is a difference in allergen uptake between keratinocytes and respiratory epithelium. Although obtained from lung epithelial cell lines, the results suggest a transcytosis of allergens by AEC, whereas in primary human keratinocytes allergens accumulate in lysosomes, a prerequisite for internal processing, and co-localize with MHC-II molecules (40). In contrast to these data, a striking specificity has been observed concerning the uptake of the major birch pollen allergen Bet-v1 by conjunctival epithelial cells of allergic individuals. Already one minute after in vivo challenge of birch pollen allergic individuals with the respective allergen a binding of Bet-v1 to conjunctival epithelial surfaces could have been observed [figure 3], whereas in healthy individuals no binding could be detected. Also the entry of Bet-v1 into the epithelial cells was evident in allergic patients, with a fast distribution, indicating rapid traffic through the epithelium, while epithelium of healthy subjects did not bind or transport Bet-v1 (41). Although the specific transport mechanism for birch pollen allergens remains to be determined, data indicate an active lipid raft and caveolae-dependent transport (42). In line with this, a major soya bean allergen has been shown to be transported through intestinal epithelial cells via a caveolae-mediated mechanism, with the allergens associating with lipid raft microdomains and an abolishment of endocytosis of the allergen after disruption of caveolae/lipid raft microdomains (43). Interestingly, the transport of birch pollen allergens seems to be allergen-specific, as grass pollen allergens do not bind to epithelial cells from birch pollen allergic individuals and vice versa, indicating an antibody-dependent process to be involved.

Antibody specific recognition c.q. uptake may not be the only mechanism by which airway epithelium may interact with allergens. As many allergens are glycoproteins, carbohydrate-recognizing pattern recognition receptors (PRR) expressed on AEC might also be involved in allergen uptake. Dectin-1 for example recognizes β(1,3)-linked glucans, carbohydrates that are found in the cell walls of plants and fungi (44), but its expression is most prominent in myeloid cells, with a rather weak expression in epithelial cells (45). The surfactant proteins (SP) A and D, which are secreted by type-II pneumocytes, belong to the family of collectins and bind to glycosylated pathogens and allergens, leading to an increased phagocytosis by alveolar macrophages (46). It has been shown that SP-D binds to different carbohydrates that can be found on the surface of subpollen particles (47). Furthermore, SP-D promotes the attachment of allergen particles to bronchial epithelial cells, while the percentage of internalized particles remains unchanged (48), which might indicate that active allergen uptake by AEC might be the rate-limiting step. Interesting data is emerging that suggest a broad role for complex carbohydrates, in particular glucans, in allergen-associated Th2 responses. For example, β-glucan structures present in the peanut allergen Ara-h1 have been shown to display Th2 inducing characteristics (49).
The fate of the allergen
When we consider the paths, which the allergen might follow, we have seen that the allergen might pass between neighboring epithelial cells or might be taken up by epithelial cells and then released again into the subepithelial layer. There, it can be taken up by tissue resident dendritic cells to start the immune response or trigger cross-linking of IgE molecules on the cell surface of mast cells and basophiles in the effector phase (50). In addition, some of the allergens find their way into the bloodstream (28, 32) of which the consequences, up to date, are not clear. To a certain degree it might not be required for the allergen to pass the epithelium as dendritic cell through cytoplasmic extensions would be able to reach into to the lumen (51, 52) and take up the allergen directly. Whether or not these extensions are presented under all conditions or whether the extensions are triggered by the interaction of allergen with the mucosal layer is unclear (53).

A second more active role of the epithelium is also possible as epithelial cells are known to express MHC class I and II molecules (54, 55) and respiratory epithelium has been reported to process and present antigens in the context of MHC II (56). Given the fact that AEC express surface molecules and growth factors that are associated with intraepithelial lymphocytes (IEL) and antigen presentation, namely IL-7, stem cell factor, ICAM-1, HLA-DR, CD40, CD80, CD86, E-cadherin (the ligand for αEβ7 integrin on IEL) or the CD2-ligand CD58 (57-61), there might be the possibility that inhaled antigens may be presented rapidly and directly via AEC to IEL within the airway mucosa. Whether or not this interaction leads to a productive immune response remains to be explored as most models seems to point towards an immunosuppressive or anergic response (62, 63).

Figure 2. Surface of the inferior turbinate of the nose. In this scanning electron microscope picture we can identify (A) the ciliated epithelial cells, (B) the microvilli carrying tops of goblet cells, (C) some undefined (inflammatory) cells attached to the surface and a wide range of yet undefined subcellular membrane structures that could represent cellular extensions of dendritic cells that reside in and underneath the epithelial layer.
Interestingly, in a recent study airway DCs were found to build clusters with nerves and proliferating T cells in a model of allergic airway inflammation, but these interactions occurred beneath the smooth muscle layer and not in the epithelium (64). However, further studies have to be performed to investigate the exact T cell subpopulation involved and the general role of AEC concerning uptake of allergens and antigen presentation within the airways, as through epithelial exosomes would be able to stimulate immune responses at a distance (65).

**Allergens affecting cellular function**

Disruption of cell-cell contacts not only allows allergens or other environmental factors to pass into local tissues, but it also affects the activity of the cells themselves (66-70). However, to a certain extent it is difficult to determine whether it is the dissociation *per se* that induces these changes. During the dissociation induced by virus local tissue cells can be activated through the presence of Toll-like receptor (TLR)-3 that recognizes viral RNA and during the dissociation induced by allergens cells can become activated through protease activated receptors (PAR) that for their stimulation depend directly on the protease activity contained with allergen extracts. So the protease activity of Der-p1 and Pen-ch13, among others, not only is responsible for the disruption of the cell-cell contacts but also for activation of epithelial cells through PARs (9, 11). We have some unpublished evidence that disruption *per se* could affect cellular activity directly, where we were able to show that disruption of cell-cell contacts in a non-enzymatic fashion induces mediators release from airway epithelial cells, although in this model the mediator release is much stronger when using an enzymatic approach that would also trigger the PAR receptors.

Recently it became apparent that the ability of allergens to bind lipids (71-74) may contribute to the uptake and/or the allergenicity of certain allergens. This exciting observation came from the work on the innate pattern recognition receptor TLR-4, where it was shown that Der-p2 was identified to resemble the critical TLR-4 co-factor MD-2 not only structurally but also functionally (75, 76). The role of MD-2 to present the lipid molecule LPS to TLR-4 can be taken over by Der-p2 and as several other members of the MD-2-like lipid-binding family are major allergens (77), this could point to a more general importance of major allergens being able to bind lipids.

This direct activation of cells by allergen applies both to epithelial cells and to tissue resident dendritic cells, which even if we would disregard the indirect effect of mediators produced by one cell on the activity of the other cell, mounts to a complex interplay between both cell types. A small but important step to tease apart the different contribution comes from the observation in mouse where we showed that the absence of TLR-4 on lung structural cells, while retaining TLR-4 on dendritic cells, abolished allergic airway inflammation caused by the HDM allergen and clearly reduced dendritic cell activation (78).

**The local microenvironment**

Up to now we have focused mostly on the direct effect of allergens on epithelial or other tissue resident cells. In this part we want to highlight some important factors that are secreted by epithelial cells and that will have an impact on immune competent in general and on dendritic cells in particular. This selection is far from exhaustive, but these factors were selected on their reported contribution to the allergic response.
Several reports have demonstrated that allergens can directly activate dendritic cells *in vitro* (79, 80). However, we should not forget that dendritic cells are active within a local tissue and that signals from the environment will reach DCs both directly and indirectly. As a consequence, the regulation of dendritic cell functions are co-dependent on the crosstalk with neighboring epithelial cells (81, 82) and the mediators they produce. The level of these mediators may themselves be affected by environmental factors and will ultimately influence the recruitment of DCs to local tissues, their activity, and even their differentiation. Recent studies have strengthened the idea that aberrant expression of genes within the epithelium might be a key driver of the inflammatory response, confirming the important role of structural cells like AEC within the initiation or treatment of complex disorders like allergy (83-91). Furthermore, secretion of CCL2 and CCL20 chemokines by *in vitro* HDM-stimulated epithelial cells was demonstrated in context of environment creation for the immature DCs chemotaxis (92). This was also seen in a model of lung epithelium exposed to the purified major allergen Phl-p1 (93). Interestingly, CCL20 release is not directly dependent on TLR2/4 triggering or PARs activation, but relies on β-glucan moieties within the HDM (94).

Airway epithelium of diseased (95) and also healthy individuals produces a variety of mediators in response to inhaled allergens that may affect the immune cells differentiation towards the allergic Th2 response. Increased baseline levels of IL-6, IL-8, GM-CSF and TNF-α were detected in epithelial cells obtained from rhinitic individuals (96) and additionally IL-6, IL-8, IL-25, IL-33 and *thymic stromal lymphopoietin* (TSLP) from healthy epithelium in response to allergens (97, 98). Expression of TSLP, a cytokine that affects DCs, B cells and Th2 cells is associated with asthma and allergic inflammatory states. One of the effects of TSLP is the induction of Th2 responses via up-regulation of OX40L on DCs, as OX40/OX40L interaction triggers and maintains the TSLP-mediated allergic reaction (99). However, another mouse model suggests that allergen induced TSLP production by nasal epithelial cells requires the additional involvement of mast cell, possibly via FcεRI, as allergen alone was not sufficient for the induction of TSLP production (100). The relative importance of TSLP was shown in a model where lung epithelium specific TSLP overexpression induced the development of an inflammatory state defined by the influx of Th2 cells and eosinophils (101). Airways inflammation triggered by DCs responding to overexpressed TSLP also led to an induction of airways hyper-responsiveness after exposure to metacholine. Moreover, it was demonstrated that TSLP may directly interact with naïve CD4+ T cells promoting their type helper 2 conversion (102).

Another cytokine produced by, among others, structural cells relevant for Th2 skewing of immune responses is **IL-25**. Similarly to TSLP, IL-25 overexpression in mouse lung epithelium leads to mucus production and airway infiltration of eosinophils and macrophages (103), typical features of the Th2 allergic inflammation. Moreover, allergen provocation in asthmatic individuals resulted in an increased production of IL-25 and its receptor in bronchial mucosa and indicated T cells, mast cells, eosinophils as potential targets and sources of this cytokines with respect to the allergic inflammation (104). Takahashi and colleagues demonstrated that IL-22 attenuates IL-25 expression in lung epithelium leading to an inhibition of allergen triggered airways inflammation, which suggests a strong and significant IL-25 involvement in allergic reactions (105).

**IL-33** (or IL-1-F11 or nuclear factor from high endothelial venules) is a cytokine released by damaged epithelium and it triggers IL-1 receptor-related protein ST2 to
promote type 2 inflammation via NF-κB/MAPK pathways (106). Little is known about IL-33, probably due to the fact that airway epithelial cells store this cytokine in nuclei, potentially as a nuclear factor (82, 107) and the mechanism of its secretion to our knowledge has not been elucidated. However, Prefontaine and colleagues (108) suggest that IL-33 levels in bronchoalveolar lavage fluid may be used as a relevant biomarker for determination of the severity of asthma. There are other studies postulating that IL-33 is sufficient and required for a severe allergic inflammation in the lung through an activation of the expression of other pro-inflammatory cytokines (109). In this recent publication (110) IL-33 rapid production in mouse lung model was demonstrated to be driven by trefoil factor 2 (TFF2). TFF2 mediator is produced among others in structural cells within hours of injury or allergen challenge (111) to enhance cell repair and it was shown to increase severity of allergic responses in lung triggered by HDM (110). Not only is then TFF2 involved in tissue repair mechanisms but also seems to play an important role in the initiation and progression of mucosal Th2 immunological responses.

Figure 3. Bet-v1 allergen binding and passing through the conjunctival epithelium obtained from an allergic individual. Double immunoTEM staining with an anti – Bet-v1 5nm gold-labeled antibody and an anti – caveolin 2 antibody labeled with 10 nm gold; 27 500 magnification. Bet-v1 is seen to strongly co-localise with a caveolar marker protein caveolin 2.

Disease affecting function
To some degree potential differences in responsiveness of cells between the diseased and healthy state could be considered a chicken or egg problem. However, even if those differences would not be the cause of the disease, they could still contribute to the expression of symptoms, their severity, or their chronicity. The first examples of differential responses that we already encountered were in the uptake of allergens by the epithelium. Not only was the allergen Bet-v1 taken up by Birch allergic individuals and not by healthy individuals, but Bet-v1 was also not taken up

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by individuals who were allergic to grass rather than birch pollen (R.R and S.T-S, unpublished observations). Even within one disease, there could be different exponents as concluded from our clinical observations in grass allergic individuals where patients with an isolated pollen allergy would respond more strongly to a nasal provocation with pollen than those with a concomitant house dust mite sensitization (112).

At the epithelial and the in vivo level we are spending a considerable effort studying the impact of HDM (84, 113-115) grass pollen allergens (93, 116) and Bet-v1 (117) using a biosystematical approach to further elucidate allergen induced responses in health and disease. A number of important observations were made. The first is that (nasal) epithelium from healthy individuals respond so strongly to allergenic triggers. From a molecular perspective this does not need to surprise us given the many mechanisms by which epithelium can detect and respond to these allergens.

However, it seems contradictory to our clinical concept where only the allergic individual responds to an allergen trigger and the healthy individual would not. This now entices the interesting questions why and where the “allergic response” peters out in a healthy individual, so that the healthy individual does not become sensitized to the allergens it is constantly exposed to. The second relevant observation indicates that some of the inflammation-mediating genes in the allergic individuals are constantly activated, which would suggest that it is the failure to down regulate these genes (rather than their initial activation) that would contribute to the allergic disease. A final example of how a disease state could contribute to the allergic response (or even the induction of new sensitization) comes from a mouse model where chronic inflammation is induced in the lung by instilling them with ex vivo activated dendritic cells (118). Long after these original dendritic cells have gone and the lung eosinophilia has perished, the mouse own lung resident dendritic cells show an activated state with increased levels of co-stimulatory molecules that are more efficient at responding to neo-allergens.

CONCLUDING REMARKS

Although we have come some way in understanding the complex interplay that occurs within local tissues we are far removed from painting the complete picture. First of all we have addressed only the epithelium and the dendritic cell, assuming that all epithelial cells are the same (which could be true or not) and that all dendritic cells are same (which is definitely not true). We have completely disregarded the contribution of fibroblasts and smooth muscle cells that seem so important in local remodeling steps seen in upper and lower airways (119, 120). Nor have we considered the role of the newly discovered innate T cells (121, 122) that are so prominently present in nasal polyps (123). These are just a few examples of factors that we have not considered at the initiation of immune responses; a situation that could be simpler than the situation during the effector phase of the immune response. Not in the last place because in the effector phase there are even more players to consider. Even in the simple situation that we have discussed here - we would need to study how our outcomes from in vivo models resemble or differ from our in vitro or ex vivo models before we can start understanding what happens in real life. The scariest realization could be that we are all individuals and that each and every one of us may respond differently, even when conditions are identical.
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