
Effects of Ole e 1 on Human Bronchial Epithelial Cells Cultured at the Air-Liquid Interface

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Airway epithelium is a highly regulated first protective barrier against inhaled substances including respiratory viruses, bacteria, air pollutants, cigarette smoke, and allergens, which have easy access to the airway mucosae [1]. For several decades, dysfunction of airway epithelium has been increasingly linked to airway inflammatory diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis [2]. Furthermore, growing evidence suggests that impaired epithelium can be the cause, rather than the consequence, of inflammatory disease [3,4] and that the nature of the host immune response is strongly determined by the state of the epithelium at the time of contact with the inhaled substances [5].

In this study, we describe the effect of Ole e 1, the main allergen of olive pollen, on bronchial epithelium throughout the differentiation process. Primary normal human bronchial epithelial cells (NHBE) from 2 female donors (age, 40 and 44 years; nonsmokers) (Lonza) were cultured at the air-liquid interface (ALI) in differentiation B-ALI-D medium (Lonza) according to the manufacturer's instructions to obtain a pseudostratified barrier with a mucociliary phenotype that represents one of the best in vitro cell models mimicking the complexity of human airway epithelium. NHBE cells were apically exposed to purified Ole e 1 (25 µg/mL) in B-ALI-D medium for 16 hours on days 7 and 21 at the ALI; these time points correspond to undifferentiated and differentiated epithelium, respectively.

Exposure of differentiating NHBE cells to Ole e 1 did not impair establishment of the epithelial barrier and its physical properties, as assessed using transepithelial electrical resistance (TEER) measurements and ultrastructural analysis based on transmission electron microscopy (TEM). No significant changes in TEER—an indirect measure of apical junctional complex (AJC) formation—were observed in response to exposure to Ole e 1 at day 7 or at day 21 at the ALI, regardless of the donor (Figure, A). Consistent with

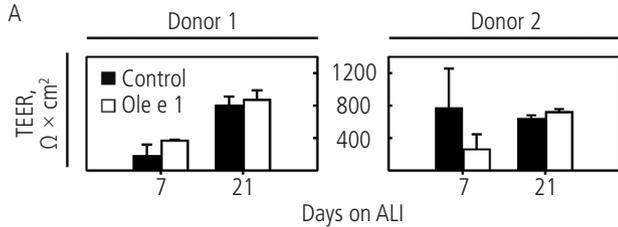


Figure A. Exposure to Ole e 1 did not impair establishment of the epithelial barrier or apical junctional complex formation, although it did alter the cytokine secretion profile in differentiating NHBE cells from 2 donors at day 7 and 21 on ALI. TEER values are the mean (SD) of triplicate determinations on a given well (n=5). TEER indicates transepithelial electrical resistance; NHBE, normal human bronchial epithelial cells.

TEER values, TEM micrographs revealed a normal epithelial structure in differentiating NHBE cells after exposure to Ole e 1 compared with untreated control cells and detected interdonor variability (Figure, B). At day 7, undifferentiated cultures showed 2-3 layers of cells with scattered microvilli at the apical surface, while at day 21 these formed a structural and functional barrier characterized by the presence of ciliated cells (note the cross-sections of the ciliary axonemes in the Figure, B) and secretory cells with a vesicle-enriched cytosol. In addition, junctional protein expression studies using immunofluorescence labeling showed that exposure to Ole e 1 did not disrupt barrier integrity, since no alterations were detected in the AJC structures in differentiating NHBE cells, apart from the variations between donors (Figure, C). At day 7 on ALI, in control and exposed NHBE cells, E-cad and ZO-1 (canonical proteins of adherens junctions [AJ] and tight junctions [TJ], respectively) were diffusely organized within the cytoplasm and at the plasma membrane, becoming localized in the plasma membrane at the cell-cell contact sites on day 21, thus indicating formation of AJCs. Western blot analysis confirmed that allergen exposure did not affect

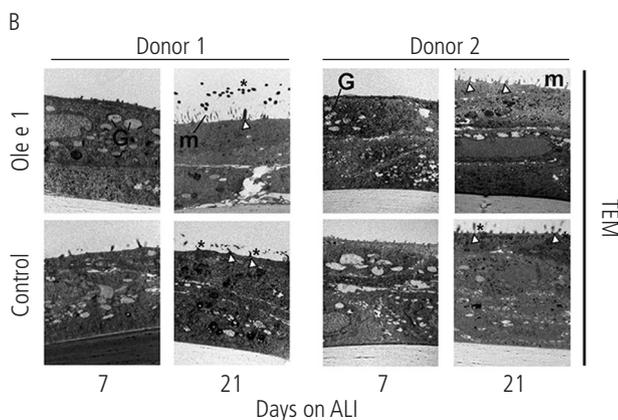


Figure B. Exposure to Ole e 1 did not impair establishment of the epithelial barrier or apical junctional complex formation, although it did alter the cytokine secretion profile in differentiating NHBE cells from 2 donors at day 7 and 21 on ALI. TEM micrographs showing cilia and the axoneme (white arrowhead), microvilli (m), and goblet (G) cells. Note the cross-sections of the ciliary axoneme (*). Magnification, $\times 10$ -20K. ALI indicates air-liquid interface; TEM, transmission electron microscopy; NHBE, normal human bronchial epithelial cells.

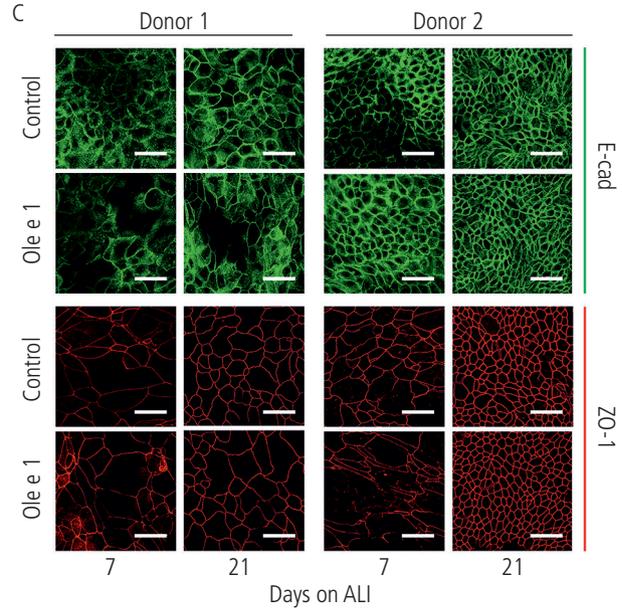


Figure C. Exposure to Ole e 1 did not impair establishment of the epithelial barrier or apical junctional complex formation, although it did alter the cytokine secretion profile in differentiating NHBE cells from 2 donors at day 7 and 21 on ALI. Confocal laser scanning microscopy analysis for E-cadherin (green) and ZO-1 (red) proteins. Representative Z-stacks are shown. Scale bar = 25 μ m. ALI indicates air-liquid interface; NHBE, normal human bronchial epithelial cells.

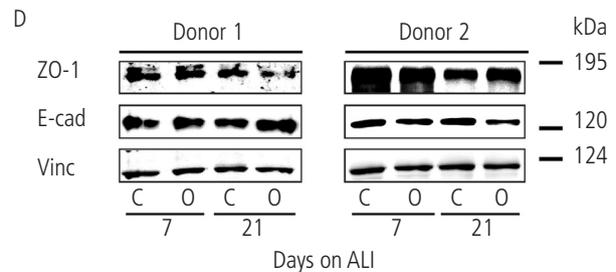


Figure D. Exposure to Ole e 1 did not impair establishment of the epithelial barrier or apical junctional complex formation, although it did alter the cytokine secretion profile in differentiating NHBE cells from 2 donors at day 7 and 21 on ALI. Western blotting analysis for the expression of E-cadherin (E-cad) and ZO-1. Ole e 1-exposed cells (O) and control cells (C). Vinculin (Vinc) was used as a lane loading control. Molecular weights of proteins are indicated in kDa. Representative Western blotting of 2 replicates is shown. ALI indicates air-liquid interface; NHBE, normal human bronchial epithelial cells.

expression of major AJ and TJ proteins in the course of NHBE differentiation (Figure, D). Interestingly, protein levels of ZO-1 and E-cadherin were similar between day 7 and day 21 of NHBE differentiation, suggesting that the reestablishment of an epithelial barrier is more likely due to the reorganization of E-cadherin and ZO-1 into AJC structures than to an increase in their levels.

Moreover, the effect of exposure to Ole e 1 on the epithelial cell populations emerging from differentiating NHBE cells at the ALI was determined using polymerase chain reaction with specific cell type markers: *CC-10* for Club cells, *FOXP3*

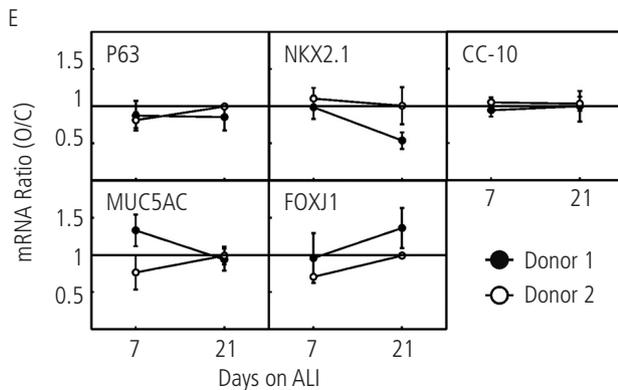


Figure E. Exposure to Ole e 1 did not impair establishment of the epithelial barrier or apical junctional complex formation, although it did alter the cytokine secretion profile in differentiating NHBE cells from 2 donors at day 7 and 21 on ALI. mRNA levels of specific epithelial cell markers after exposure of NHBE cells to Ole e 1 (O) compared with control cells (C). Relative mRNA levels of CC-10 (Club cell), FOXJ1 (ciliated cells), MUC5AC (goblet cells), NKX2.1 (progenitor cells), and P63 (basal cells) were determined by semiquantitative reverse transcription polymerase chain reaction and expressed as a fold-change of control values after normalization using GAPDH as a housekeeping control. A ≥ 2 -fold change was considered significant. Data shown are mean (SD) of 3 replicates. ALI indicates air-liquid interface; NHBE, normal human bronchial epithelial cells.

for ciliated cells, *MUC5AC* for goblet cells, *NKX2.1* as a lung-committed marker, and *P63* for basal cells (Figure, E). In control NHBE cells, the expression levels of *FOXJ1*, *MUC5AC*, and *NKX2.1* mRNA changed during the course of differentiation at the ALI. We did not observe significant alterations in any of the transcript levels studied upon exposure to Ole e 1, except for the decrease in *NKX2.1* mRNA levels at day 21 at the ALI in donor 1.

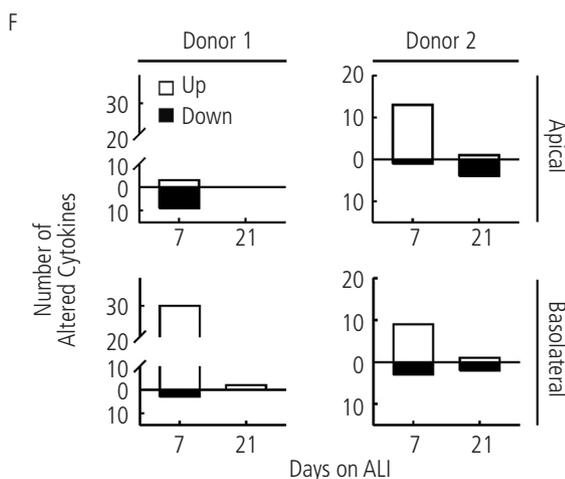


Figure F. Exposure to Ole e 1 did not impair establishment of the epithelial barrier or apical junctional complex formation, although it did alter the cytokine secretion profile in differentiating NHBE cells from 2 donors at day 7 and 21 on ALI. Cytokine secretion profile as determined by antibody microarray analysis. Bar graphs indicate the number of significantly altered cytokines that displayed ≥ 2 -fold changes compared with control cells in the apical and basolateral compartments of NHBE cultures. NHBE indicates normal human bronchial cells; TEER, transepithelial electrical resistance; TEM, transmission electron microscopy; ALI, air-liquid interface.

Finally, a RayBio Human Cytokine Antibody Array 5 (RayBiotech) analyzed with the GenePix Pro 7.1 software (Molecular Devices) was used to assess whether exposure to Ole e 1 affected the levels of cytokines secreted from differentiating NHBE cells (Figure, F). Interestingly, the number of altered cytokines secreted by undifferentiated cells (day 7 at the ALI) exposed to Ole e 1 was higher when compared with differentiated NHBE cells (day 21 at the ALI), which showed only a few modified mediators from both the apical and the basolateral sides of the culture, regardless of the donor (Online Supplementary Tables S1, S2, and S3). In general, undifferentiated cells displayed a greater number of upregulated cytokines on the basolateral side than on the apical side. These included cytokines that have been previously described in asthma and allergic inflammation. For example, there was a 7.82-fold increase in apical secretion of IP-10/CXCL10 in donor 1. Basolateral secretion of TARC/CCL17 was also upregulated (2.25-fold increase) in this donor. In contrast, the apical release of MCP-1 was upregulated (4.83-fold increase) after exposure to the allergen in donor 2. Thus, the altered cytokine profile obtained showed strong dependence on the donor. Only 3 cytokines were found to be shared by donor 1 and donor 2. IGFBP4 was upregulated, especially on the basolateral side (4.98-6.46-fold increase). Leptin secretion was upregulated on the basolateral and apical sides of donor 1 and donor 2, respectively; in contrast, MIG/CXCL9 was downregulated (0.29-0.39 fold increase) on the apical side. This finding suggests that cytokine outcomes may be related to the cellular state of differentiation at the time of allergen exposure, possibly owing to the significant changes in gene expression that take place during the differentiation process of NHBE cells [6,7]. The heterogeneity observed on the cytokines secreted by airway epithelial cells upon interaction with an allergen would provide a variety of microenvironments that support the activation/maturation of various immune cells and, consequently, the final host immune response. In addition, important differences in cytokine patterns were also observed between the 2 donors, both in the number and in the type of cytokine secreted, indicating that the response was also strongly influenced by donor features, as indicated in previous reports [8,9]. Interdonor variability is one of the main disadvantages of using primary NHBE cells cultured at the ALI for research, as it hinders interpretation of results. Thus, our study supports the idea that both the donor and the state of differentiation of bronchial epithelial cells have a major influence on the immune response to Ole e 1.

Disruption of airway epithelial AJCs by environmental proteases could facilitate penetration of the allergen through the epithelium and access to immune cells, thus contributing to the initiation and exacerbation of allergic diseases. Given that Ole e 1, which is not a protease, stimulated NHBE cells to release cytokines without disrupting epithelial integrity, it is likely that the mechanism involved in the sensitization and/or the development of allergic reactions to this allergen is protease-independent. A protease-independent mechanism has been described for several allergens, including allergens from pollens and house dust mite [10].

In this study, we demonstrated that Ole e 1 allergen induced the release of a plethora of cytokines from NHBE cells

cultured at the ALI, which is affected both by the differentiation status of the cells at the time of exposure and donor features. Additionally, our findings highlight the importance of using a well-characterized model of human airway for allergy-related research.

Supplementary data

Supplementary data are available at Journal of Investigational Allergology and Clinical Immunology online.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Previous Presentations

Some of the data from this study were presented in poster format at the European Academy of Allergy and Clinical Immunology (EAACI) Congress, Vienna, Austria. June 2016

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