Epithelial Permeability to Ole e 1 Is More Dependent on the Functional State of the Bronchial Epithelium Than on the Activity of Der p 1 Protease Acting as an Adjuvant to the Bystander Allergen

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A dysfunctional epithelial barrier has been widely associated with allergy [1], although it is not clearly established whether this dysfunction is the cause or a consequence of the disease. Moreover, airway epithelial barrier integrity can be impaired by environmental proteases—mostly allergens—derived from a wide variety of biological sources such as mites, cockroaches, food plants, fungi, and pollens [2,3]. These allergens include cysteine proteases, which are potent adjuvants for promoting type 2 helper T-cell immune responses in the airways. Although it is well known that the cysteine protease Der p 1, a major allergen from house dust mite, exerts various effects on the airway epithelium that could contribute to allergic airway diseases [4-6], the cleavage of tight junction (TJ) proteins was the first activity described for it [5]. Der p 1 also cleaves several molecules involved in the immune response, including CD23, CD25, and IL33-alarmin [4,6]. The identification of new targets for Der p 1 could be important when defining the molecular mechanisms for the initiation and exacerbation of respiratory allergies after allergen exposure.

In the present study, we analyzed the effect of Der p 1 cysteine protease activity on bronchial epithelial permeability to Ole e 1, the main allergen of olive pollen [7]. Exposure of air–liquid interface (ALI)–cultured Calu-3 cells to Der p 1 for 24 hours decreased transepithelial electrical resistance (TEER) values and promoted a discontinuous staining pattern for ZO-1 (TJ) in an epithelial state-dependent manner: this occurred on day 2 of culture, when the functional barrier was still being established, but not on day 7 when the barrier was well-formed (Figure, A, Fig.S1, and Fig. S2A). Interestingly, TEER values were restored to control values 10 minutes after removal of protease. While it is well documented that Der p 1 can disrupt the airway epithelial barrier by cleaving apical junctional

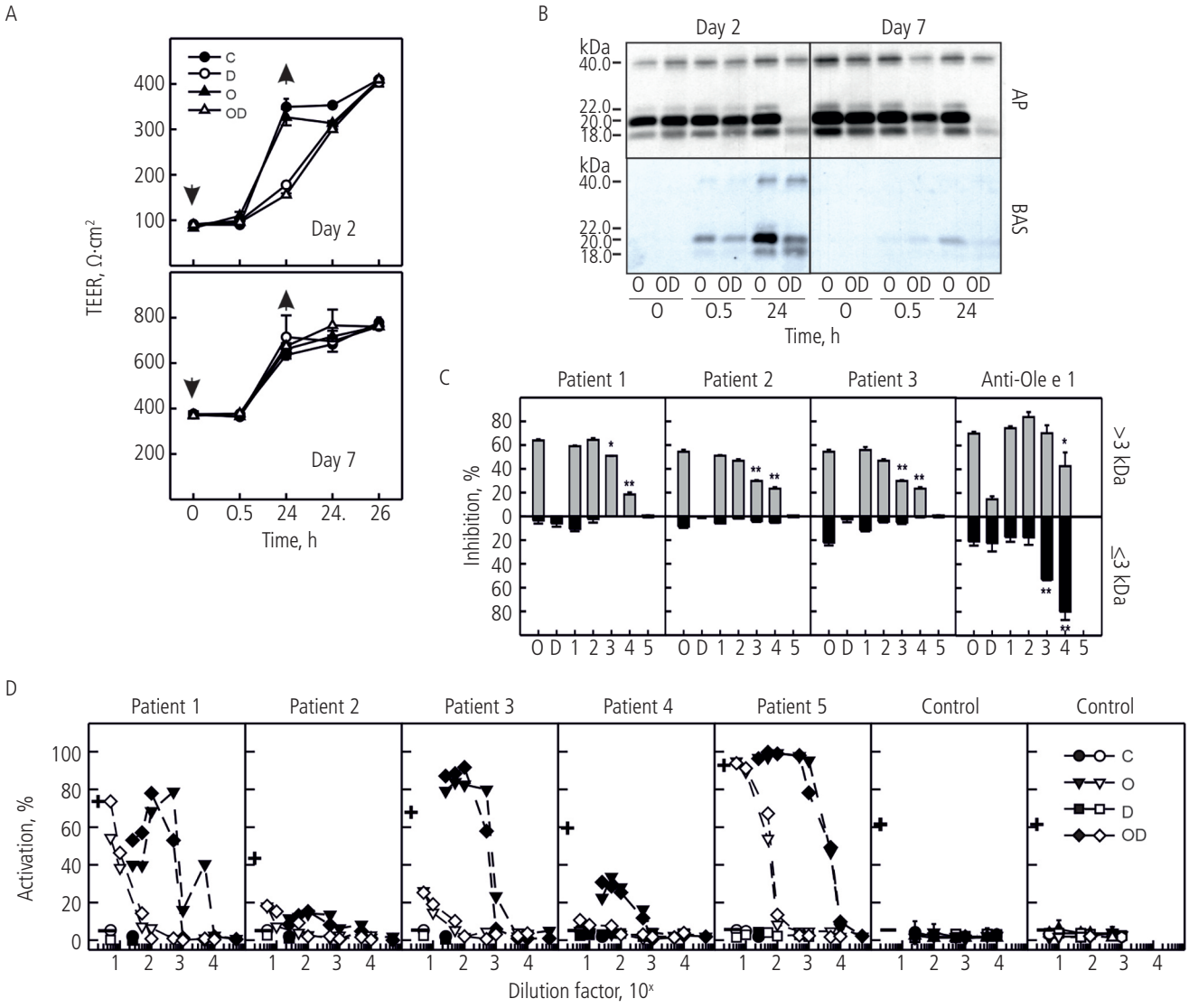

Figure. Effect of Der p 1 coexposure on the response to Ole e 1 of ALI-cultured Calu-3 cells on days 2 and 7. A, Time course of TEER values of ALI-cultured Calu-3 cells exposed to Ole e 1 and/or Der p 1 compared with unexposed cells. TEER was measured at the indicated time points and shown as the mean (SD) value of triplicate determinations. Down and up arrows indicate the time of addition and removal of the allergen, respectively. B, Effect of Der p 1 coexposure on bronchial epithelial permeability to Ole e 1. ALI-cultured Calu-3 cells were coexposed to Ole e 1 and Der p 1 on days 2 and 7, and the presence of Ole e 1 was determined by immunoblotting in the medium at different time points (0 hours, 0.5 hours, and 24 hours). Molecular masses of Ole e 1 forms in kDa are indicated: 40, dimer; 22, hyperglycosylated; 20, glycosylated; and 18, nonglycosylated. For immunological characterization, Ole e 1–derived products resulting from Der p 1 cleavage were separated into 2 fractions by Nanosep-3K: >3 kDa and ≤3 kDa. C, Inhibition ELISA of Ig binding to Ole e 1-coated-wells using cleavage products as inhibitors (1 μg/mL) and serum IgE from 3 olive pollen–allergic patients (Table S1) or IgG from specific polyclonal anti-Ole e 1 antiserum. Data are expressed as inhibition percentage (%) with respect to the control and shown as the mean (SD) of duplicate determinations. Protease-treatments: 1, 0 hours; 2, 0.5 hours; 3, 8 hours; and 4, 24 hours; and 5, HCl-hydrolyzed Ole e 1. O indicates Ole e 1; D, Der p 1. Significant differences: **P*<.05, ***P*<.01. D, Analysis of basophil activation by flow cytometry in Ole e 1–allergic patients (but not to Der p 1, n=5,) compared with the control group (n=5, the average response is shown) (Table S1). Cells were stimulated with different doses of >3 kDa (black symbols) and ≤3kDa (white symbols) fractions obtained from phosphate-buffered saline (C), Ole e 1 (O), Der p 1 (D), and Ole e 1–derived products resulting from Der p 1 cleavage (OD). Data are given as the percentage (%) of activation (CD63 expression). +, anti-IgE control; –, basal control. AP indicates apical medium; BAS, basolateral medium; O, exposure to Ole e 1; OD, exposure to Ole e 1 in combination with Der p 1.

complex proteins (including ZO-1) [8], the novelty of our findings is the dependence on functional state of the airway barrier disruption caused by Der p 1. In this sense, López-Rodríguez et al [9] found that Ole e 1 induced cytokine release from ALI-cultured NHBE cells in a differentiation state-dependent manner. As previously reported, Ole e 1 altered neither TEER values nor the ZO-1 staining pattern over time. ALI-cultured cells were then apically exposed to Ole e 1 on days 2 and 7, and the presence of the allergen was analyzed on the basolateral medium at different time points by immunoblotting (using an anti-Ole e 1 antiserum) to determine whether the protease could facilitate the passage of the allergen across the cell layer, a requirement for allergen sensitization [10] (Figure, B).

Despite the potential role of protease activity in allergen sensitization indicated by numerous studies, we demonstrated that epithelial permeability to Ole e 1 was more dependent on the functional state of the epithelium than on protease activity. The passage of the allergen across the epithelial barrier was higher on day 2 than on day 7 in ALI—with or without the protease—as indicated by its detection on the basolateral side after 0.5 hours of culture, and its levels increased over time. The SDS-PAGE pattern is characterized by the 18-kDA band (nonglycosylated form) and the 20-kDa band (glycosylated form) [7]. Again, our data provide evidence that the functional state of the epithelium at the time of contact plays a key role in its response to the allergen.

Both the remarkable decrease in Ole e 1 levels on the apical side and the alteration of its electrophoretic pattern after 24 hours with Der p 1 suggest proteolytic cleavage by this protease. Therefore, Ole e 1 was incubated with the protease for 24 hours, and aliquots were taken at different time points and analyzed using mass spectrometry (MS) and immunoblotting (Fig. S2B). In the absence of protease, the allergen-MS spectrum had 2 majority peaks; with Der p 1, the intensity of the 17 518.4 m/z peak (monoprotonated species of the glycosylated form) decreased over incubation time as both the 16 197.4 m/z peak (monoprotonated species of the nonglycosylated form) and the <12 500 m/z area increased. Immunoblot analyses supported MS data, indicating that Ole e 1 is a novel target of Der p 1. Edman degradation assays indicated that Der p 1 removed the first 10 amino acid residues of Ole e 1 after 0.5 hours of treatment: the N-terminal sequences obtained were EDVP and FHIQ (Fig. S2B). Although the N-terminal of Ole e 1 does not display a Der p 1 consensus cleavage sequence [11], the allergen was efficiently cleaved by this protease and, after 24 hours' exposure, was degraded into short peptides. These findings suggest that other factors might be involved in cleavage by Der p 1 [11].

Finally, inhibition ELISA showed that both ≤3-kDa and >3-kDa fractions obtained by ultrafiltration of Ole e 1 cleavage products were able to bind specific IgG (rabbit antiserum), although only the >3-kDa fraction exhibited IgE reactivity with sera from 3 olive pollen–allergic patients (Figure, C, Table S1). Moreover, both fractions activated basophils from 3-4 out of 5 patients tested in a dose-dependent manner (Figure, D). The absence of IgE reactivity in the ≤3-kDa fraction could be explained in part by the sensitivity of the assay, since this fraction contained peptides with appropriate molecular masses for activating basophils from allergic patients, albeit at lower levels. Our data were supported by identification of IgG and IgE epitopes in Ole e 1 based on using short overlapping synthetic peptides covering the full polypeptide chain [12]. The cleavage of Ole e 1 by Der p 1 may also generate T-cell peptides that mediate immunomodulation of the host response. In this sense, Wildner et al [13] reported that peptides derived from Ole e 1 cleavage by cathepsin S (an endolysosomal cysteine protease) substantially overlapped with T-cell epitopes of this allergen [14].

In conclusion, we show that epithelial permeability to Ole e 1 is more dependent on the functional state of the bronchial epithelium than on Der p 1 protease activity. The finding that Ole e 1–derived peptides by Der p 1 cleavage effectively bind to IgG and IgE and activate basophils from olive pollen–allergic patients suggests an additional mechanism by which environmental proteases may facilitate sensitization to other allergens, acting synergistically with their ability both to disrupt the epithelial barrier and to activate epithelial cells. In this sense, Der p 1 has been recognized as an "initiator allergen" of IgE sensitization, since it exhibits the properties required to promote an allergic response to both itself and other unrelated allergens [15]. This study highlights a new understanding of the early events that could contribute to allergy in humans.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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