

***Dermatophagoides pteronyssinus* and bioelectric properties of airway epithelium: role of cysteine proteases**

N. Roche*, T.C. Chinet*, N-E. Belouchi*, C. Julié[#], G.J. Huchon*

Dermatophagoides pteronyssinus and bioelectric properties of airway epithelium: role of cysteine proteases. N. Roche, T.C. Chinet, N-E. Belouchi, C. Julié, G.J. Huchon. ©ERS Journals Ltd 2000.

ABSTRACT: Several epidemiological studies suggest that exposure to house dust mite allergens plays a role in the pathogenesis of asthma. Since many of these allergens exhibit enzymatic properties, they may damage the airway epithelium.

To characterize the effects of low doses of *Dermatophagoides pteronyssinus* on the airway epithelium, the effect of *D. pteronyssinus* on the epithelial bioelectric properties of tracheal fragments of non-sensitized Lewis rats was studied, using Ussing-type chamber technique.

The addition of a crude *D. pteronyssinus* extract containing 20 µg·mL⁻¹ of *Der p1* allergen in the presence of 1.5 mM dithiothreitol (DTT, an activator of cysteine proteases), induced a progressive increase in bioelectrical conductance (+ 12.0±1.5%, n=12, p<0.005), an index of epithelial permeability, without affecting the short circuit current (which reflects active ion transports). The *D. pteronyssinus*-induced increase in epithelial conductance was related to the cysteine-protease activity of the allergen since it was not observed in the absence of DTT (n=12), and was completely suppressed in the presence of 10 nM E-64, a specific inhibitor of cysteine proteases (n=12). *D. pteronyssinus*-induced increase in epithelial conductance could be entirely attributed to an increase in the paracellular conductance (+ 11.2±1.2%, n=8, p<0.01). There was no electrophysiological evidence of rupture in epithelial continuity, and no cell detachment was observed on microscopic examination.

In conclusion, the cysteine protease activity of crude *Dermatophagoides pteronyssinus* extract is able to increase the epithelial paracellular conductance of rat tracheal tissues, even at relatively low doses that do not induce cell detachment or cell death. *Eur Respir J* 2000; 16: 309–315.

*Laboratoire de Biologie et Pharmacologie des Epithéliums Respiratoires, and [#]Laboratoire d'Anatomo-pathologie, Université de Paris René Descartes, Hôpital Ambroise Paré, France.

Correspondence: G.J. Huchon, Service de Pneumologie et Réanimation, Hôpital de l'Hôtel Dieu, 1 place du Parvis, Notre Dame, F-75004 Paris, France. Fax: 33 142348448.

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Occurrence of asthma is considered to depend on both genetic predisposition and exposure to environmental factors [1]. More than 90% of asthma cases are associated with an atopic status, and 60–100% of asthmatics are sensitized to house dust mites (HDM). In addition to their immunoglobulin(Ig)E-dependent effects [2–5], allergens of *Dermatophagoides* spp. may affect the airway mucosa through enzymatic mechanisms, since some of them are digestive enzymes of the mite with cysteine protease and serine protease activities [6, 7]. Nine groups of allergens of the HDM *Dermatophagoides pteronyssinus* have been identified so far, and at least four have enzymatic properties which may enable allergens to cross the epithelial barrier and reach antigen-presenting cells [6, 7].

Therefore, several studies recently focused on the effects of enzymatic activities of *D. pteronyssinus* on the epithelium [8–12]. The cysteine protease activity of *D. p1* increases bovine bronchial mucosal permeability to albumin and is capable of causing cell detachment of cultured Madin-Darby canine kidney (MDCK) cells and canine tracheal epithelial cells [8]. In this study, the increase in epithelial permeability of bronchial fragments was observed at relatively high concentrations of highly purified

allergen (300 µg·mL⁻¹ *Der p1*), which may not be relevant with respect to usual exposure levels [8]. In addition, while the increased permeability to albumin was likely due to an effect on paracellular pathways, an effect on transcellular transports can not be ruled out since active transport of albumin has been described in animal airways [13]. In a subsequent study, it has been demonstrated that both the cysteine protease and serine protease fractions of *D. pteronyssinus* induce an increase in epithelial paracellular permeability to mannitol, which is related to tight junction breakdown and is associated with initiation of cell death [10]. However, in a more recent study *D. pteronyssinus* cysteine protease but not serine proteases altered tight junctions, allowing the allergen to cross the epithelial layer [12]. These two studies were performed on epithelial monolayers, in which the characteristics of epithelial cells and junctions may not entirely reflect that of the airway epithelium *in vivo*. In addition, the use of purified protease fractions in the studies by HERBERT *et al.* [8] and WINTON *et al.* [9–10] makes it difficult to determine which enzymatic activity is the most relevant, and which concentrations of inhaled allergens are required to achieve such effects *in vivo*. This

point was addressed by WAN *et al.* [12], who described a method for calculation of relevant *D. pteronyssinus* concentrations, based on the daily amount of inhaled mite faecal pellets and the area of exposed bronchial mucosa. Finally, serine protease and metalloprotease activities of *D. pteronyssinus* have been shown to induce cell detachment in epithelial cell lines, together with a release of cytokines which was observed at allergen concentrations of $10 \mu\text{g}\cdot\text{mL}^{-1}$, while cell detachment occurred at $40 \mu\text{g}\cdot\text{mL}^{-1}$ [11]. Surprisingly, these authors did not find any evidence of such effects of the cysteine protease activity of *Der pI* [11], which represents at least half of *D. pteronyssinus* allergenic content [6, 7].

Considering the questions raised by the above-mentioned data, the authors wondered whether the cysteine protease activity, contained in low doses of crude allergen extracts, affects the epithelial paracellular and/or transcellular bioelectrical conductance of intact airway tissues. Since electrophysiological techniques based on Ussing-type chambers allow highly sensitive measurements of epithelial permeability and permit separate assessment of paracellular and transcellular pathways, rat tracheal fragments mounted in Ussing-type chambers were used to: 1) measure the effects of low doses of *D. pteronyssinus* extracts on airway bioelectrical conductance, an index of epithelial permeability; 2) determine whether the observed effects could be ascribed to the cysteine-protease activity of the major allergen *Der pI*; and 3) assess whether the effect of *D. pteronyssinus* on airway epithelial permeability is due to an effect on the transcellular transports or on the paracellular pathway. The effect of *D. pteronyssinus* on airway morphology was also studied using light microscopy.

Materials and methods

Animals

Nonsensitized male Lewis rats, weighing 275–300g, were obtained from Charles River (Saint-Aubin-Lès-Elbeuf, France). The rats were housed in a positive pressure unit protected by high-efficiency particulate air (HEPA) filters (Iffa Credo, Saint-Germain sur L'Arbresle, France) under constant temperature and day-night lighting, and fed with standard rat diet (Ets L.Pièremont, Provins, France). The enzymatic effect of *D. pteronyssinus* allergens on airway epithelial bioelectric variables was characterized in 60 rat tracheal fragments obtained from 30 rats. Eight additional tracheas were used to determine whether *D. pteronyssinus* exerted its effect on transcellular or paracellular pathways. Animals were handled according to the Helsinki Convention for the Use and Care of Laboratory Animals.

Allergens

A crude *D. pteronyssinus* extract was used (Stallergènes Laboratories, Antony, France). This extract was titrated according to the concentration of *Der pI*, which represents half of the total allergen content. The stock solution contains $200 \mu\text{g}\cdot\text{mL}^{-1}$ of *Der pI*, diluted in the spent growth

medium of the mite. A solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ of *Der pI* was obtained by diluting 1 mL of the stock solution in 9 mL of Krebs' Bicarbonate Ringer (KBR).

Measurement of epithelial bioelectric properties

The animals were anaesthetised by intraperitoneal injection of $80 \text{mg}\cdot\text{kg}^{-1}$ of sodium thiopental and killed by bleeding. The trachea was isolated from surrounding tissues, removed and immersed in 37°C KBR gassed with oxygen (O_2) (95%) and carbon dioxide (CO_2) (5%). It was opened longitudinally by cutting the anterior wall, and mounted in Ussing-type chambers bathed with circulating 37°C KBR bubbled with 95% O_2 –5% CO_2 . The surface of exposed tissue was 63.6mm^2 .

The Ussing-type chambers were connected to a voltage clamp system (DVC1000, World Precision Instruments, Aston, UK) with one pair of voltage-sensing electrodes and one pair of current-passing electrodes; all electrodes consisted of 2.5% agar 3M KCL bridges. The transepithelial potential difference (PD) and the short-circuit current (I_{sc}) were measured. The transepithelial bioelectrical conductance (G) was determined by clamping the PD to +10 mV, recording the deflection in I_{sc} , and applying Ohm's law. To verify that Ohm's law ($\text{PD} = I/G$) could be used for the calculation of the conductance under our experimental conditions, we measured the intensity of the current required to clamp tissue voltage at -10, -5, 0, +5, and +10 mV in six rat tracheal fragments.

Experimental protocols

For each experiment, the tissues were allowed to equilibrate in the chamber for 30 min before addition of agents (baseline period). G was measured every 20 s; I_{sc} was monitored continuously and PD was recorded every 10 min. The experimental protocol was initiated when electrophysiological criteria of viability and stability were present, *i.e.* $\text{PD} \geq -0.5 \text{mV}$, $I_{sc} > 0.5 \mu\text{A}\cdot\text{cm}^{-2}$, $G \leq 40 \text{mS}\cdot\text{cm}^{-2}$ and variation in $G < 10\%$ during the 30-min baseline period [14]. Then agents were added to the apical solution, while the same volume of KBR (*i.e.* 0.1–1 mL) was simultaneously added to the basolateral solution, to keep the hydrostatic pressure across the tissue constant. Measurements were continued for 30 min after the addition of agents.

Enzymatic effect of D. pteronyssinus allergens on airway bioelectric properties. The following agents and concentrations were added to the 10 mL apical solution: *D. pteronyssinus* solution containing 2 or $20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI* plus 1.5 mM dithiothreitol (DTT, an activator of cysteine-proteases); *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI* without DTT; and *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI* plus 1.5 mM DTT in the presence of 10 nM L-trans-Epoxy succinyl-leucylamido(4-guanidino)butane (E-64, an inhibitor of cysteine proteases). The medium used to grow mites plus 1.5 mM DTT was used as a control solution. The effect of each of these solutions was studied in 12 rat tracheal fragments. Since preliminary observations showed that the effect of *D. pteronyssinus* allergens on the bioelectrical conductance of the epithelium was a slow process, the effect of each

solution was assessed by comparing the change in bioelectric variables during the 30-min test period to the change during the 30-min baseline period. To take baseline bioelectric characteristics into account, all data were expressed as percentages of the initial baseline value.

Calculation of transcellular and paracellular conductances. Paracellular conductance (G_{para}) was determined using amiloride, as described by YONATH *et al.* [15]: addition of increasing concentrations of amiloride to the apical solution inhibits the apical membrane's Na^+ conductance, and therefore decreases I_{sc} and G . when G is plotted against I_{sc} for each concentration of amiloride, the extrapolated G for $I_{sc}=0$ can be calculated, and represents G_{para} . The transcellular conductance (G_{cell}) can then be calculated from G and G_{para} ($G=G_{cell} + G_{para}$) [15]. For each sample, it was ensured that conductance was stable during a 30-min control period, which was required for the subsequent study of *D. pteronyssinus* effect. Increasing concentrations of amiloride (*i.e.* 10^{-5} , $2 \cdot 10^{-5}$, $4 \cdot 10^{-5}$, and 10^{-4} M) were added to the mucosal bath and, for each concentration, G was plotted against I_{sc} to allow calculation of G_{para} and G_{cell} . The tissue was then washed several times with amiloride-free KBR and allowed to equilibrate during 30 min; after verification that G had returned to baseline pre-amiloride value, the control solution (*D. pteronyssinus* growth medium+DTT) was added to the apical solution, and bioelectric variables were measured during 30 min. Finally, the same increasing doses of amiloride were again added for calculation of G_{para} and G_{cell} . The effect of *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI and 1.5 mM DTT was then studied using the same method. Thus, for each sample G_{para} and G_{cell} could be calculated before and after addition of the control solution, and before and after addition of *D. pteronyssinus* and DTT.

Solutions and reagents

KBR was composed of (in mM) Na 140, Cl 120, K 5.2, HCO_3 25, H_2PO_4 0.4, HPO_4 2.4, Ca 1.1, Mg 1.2, and glucose 5 (pH 7.3). E-64 stock solution ($2 \cdot 10^{-5}$ M) was obtained by solubilization of 3.6 mg E-64 in 500 mL deionized distilled water. DTT stock solution (30 mM) was obtained by solubilization of 2.3 mg DTT in 500 μL KBR. Amiloride solutions were obtained by solubilization of the reagent in deionized distilled water (stock solution: $5 \cdot 10^{-3}$ M). Agar was prepared by solubilization of 2.5 g agar in 100 mL of a 3M KCl solution. Control mite culture medium was obtained by solubilization of 2 mg of lyophilized medium in 20 mL of $4 \text{ g}\cdot\text{L}^{-1}$ ammonium bicarbonate at 37°C , centrifugation of that solution at 2,000 rpm for 15 min at 30°C , and filtration. All solutions and reagents were obtained from Sigma-Aldrich (L'Isle D'Abeau Chesnes, France), except mite culture medium and allergens which were from Stallergaenes laboratories, Fresnes, France.

Histological examination of tracheal fragments

Immediately after completion of electrophysiological experiments, 6 tracheal fragments exposed to the *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI and 1.5 mM DTT and six, exposed to control solution, were

removed and fixed in Bouin solution. They were then embedded in paraffin, cut in $5 \mu\text{m}$ sections, and stained with haematoxylin and eosin. Three sections of each fragment were examined by an independent observer, who was unaware of the agent to which the tissue had been exposed and how the bioelectric variables of the tissue had been affected. The following patterns were scored semi-quantitatively (from 0–4): epithelial cell detachment, epithelial intercellular disruption, mucosal infiltration by inflammatory cells, and mucosal oedema.

Statistical analysis

All data are reported as means \pm SEM. Paired t-test was used for comparing changes in bioelectric variables during the test period to changes during the baseline period. Analysis of variance was used to compare the effects of tested solutions, the outcome variable being the difference between changes in bioelectric variables during baseline and test periods. Histological scores were compared using nonparametric frequency analysis. A value of $p < 0.05$ was considered as significant.

Results

Applicability of Ohm's law and baseline variables

The relationship between the intensities required to clamp tissues at -10, -5, 0, +5, and +10 mV and these voltages was linear ($r^2=0.94$; $p < 0.005$), which allowed us to use the Ohm's law for calculation of G according to the following equation:

$$G = \Delta I / \Delta V = (I_{10\text{mV}} - I_{sc}) / (10 - 0) \quad (1)$$

The baseline bioelectric variables of tracheal tissues are shown in table 1. There was no difference between conditions ($p > 0.05$ for each comparison).

Enzymatic effect of *D. pteronyssinus* allergens on airway bioelectric properties

The addition of the *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI with 1.5 mM DTT induced a significant increase in G , as shown in fig. 1 ($p < 0.005$). Other tested solutions (*i.e.* solutions containing $2 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI + DTT, $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI without DTT, $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI + DTT in the presence of E-64, and control solution) had no significant effect on conductance ($p > 0.05$ for each comparison). The effect of the *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI + 1.5 mM DTT was different from that of other solutions ($p < 0.001$). There was no significant effect of any solution on PD and I_{sc} (fig. 2).

Effect of *D. pteronyssinus* allergens on transcellular and paracellular conductances

As assessed by experiments using amiloride, the addition of the control solution had no effect while *D. pteronyssinus* solution containing $20 \mu\text{g}\cdot\text{mL}^{-1}$ Der pI with 1.5

Table 1. – Bioelectric variables (mean±SEM) in test groups

Tested solution	Start of baseline period	End of baseline period	Start of test period	End of test period
<i>G</i> mS·cm ⁻²				
<i>D. pteronyssinus</i> [#] + DTT [‡]	33.7±1.7	34.8±1.9	34.8±1.9	35.9±1.9
<i>D. pteronyssinus</i> [¶] + DTT [‡]	22.7±2.7	23.3±3.0	23.1±2.9	26.0±3.4*
<i>D. pteronyssinus</i> [¶]	27.0±3.2	27.3±3.9	27.3±3.9	26.8±4.7
E-64 (10 nM) + <i>D. pteronyssinus</i> [¶] + DTT [‡]	30.2±2.4	30.0±2.5	28.3±2.5	29.0±2.8
Control solution (growth medium + DTT [‡])	28.3±1.7	28.8±3.1	28.7±3.3	29.9±3.7
<i>I</i> _{sc} µA·cm ⁻²				
<i>D. pteronyssinus</i> [#] + DTT [‡]	37.1±3.4	39.1±4.6	35.9±5.2	34.5±5.0
<i>D. pteronyssinus</i> [¶] + DTT [‡]	36.4±7.4	38.5±8.3	30.5±7.7	34.5±7.7
<i>D. pteronyssinus</i> [¶]	37.8±6.7	41.2±10.6	43.1±10.8	52.4±12.5
E-64 (10 nM) + <i>D. pteronyssinus</i> [¶] + DTT [‡]	31.1±5.8	38.8±6.4	37.8±7.2	43.1±7.8
Control solution (growth medium + DTT [‡])	33.8±5.4	37.8±8.2	35.1±7.8	35.8±7.8
PD (mV, negative)				
<i>D. pteronyssinus</i> [#] + DTT [‡]	1.1±0.1	1.2±0.2	0.7±0.2	0.6±0.2
<i>D. pteronyssinus</i> [¶] + DTT [‡]	1.5±0.3	1.6±0.3	1.2±0.3	1.2±0.2
<i>D. pteronyssinus</i> [¶]	1.5±0.3	1.6±0.4	1.6±0.4	1.8±0.4
E-64 (10 nM) + <i>D. pteronyssinus</i> [¶] + DTT [‡]	1.0±0.2	1.3±0.2	1.3±0.2	1.6±0.3
Control solution (growth medium + 1.5 mM DTT [‡])	1.3±0.2	1.4±0.4	1.1±0.3	1.3±0.4

n=12 for each condition. [#]: 2 µg·mL⁻¹ *Der pI*; [¶]: 20 µg·mL⁻¹ *Der pI*; *: p=0.0001 when compared to baseline of test period. *G*: bioelectrical conductance; *I*_{sc}: short cut impedance; PD: potential difference; *D. pteronyssinus*: *Dermatophagoides pteronyssinus*; DTT: dithiothreitol; *Der pI*: *D. pteronyssinus* allergen I; [#]: 2 µg·mL⁻¹ *Der pI*; [¶]: 20 µg·mL⁻¹ *Der pI*; [‡]: 1.5 mM DTT.

mM DTT induced an increase in *G* and *G*_{para} (p<0.05 and p<0.01, respectively), without affecting *G*_{cell} (fig. 3).

Der pI plus 1.5 mM DTT and fragments exposed to control solution (p>0.05 for each comparison).

Histological examination of tracheal fragments

Epithelial cell detachment, epithelial intercellular disruption, mucosal infiltration by inflammatory cells, and mucosal oedema did not differ between fragments exposed to the *D. pteronyssinus* solution containing 20 µg·mL⁻¹

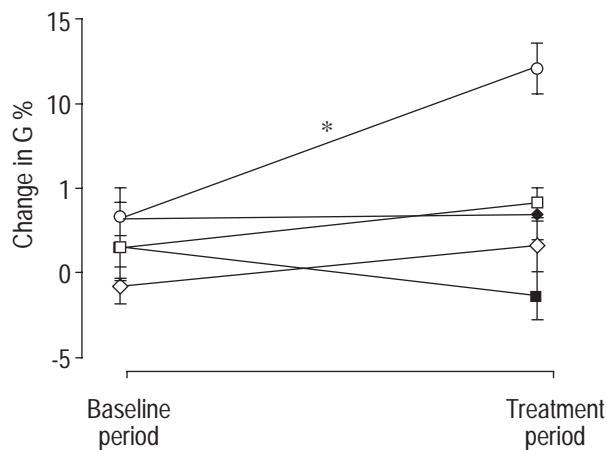


Fig. 1. – Change in conductance *G*, expressed as the percentage of the initial baseline value, during the 30-min baseline period and the 30-min test period. n=12 for each condition. *D. pteronyssinus*: *Dermatophagoides pteronyssinus*; (○): *Dermatophagoides pteronyssinus* (20 µg·mL⁻¹ *Der pI*) + DTT (1.5 mM); (●): *Dermatophagoides pteronyssinus* (20 µg·mL⁻¹ *Der pI*) + DTT (1.5 mM); (◆): *Dermatophagoides pteronyssinus* (20 µg·mL⁻¹ *Der pI*) without DTT; (◇): *Dermatophagoides pteronyssinus* (20 µg·mL⁻¹ *Der pI*) + DTT (1.5 mM) in the presence of E64 (10 nM); (□): control, i.e. mite growth medium + DTT (1.5 mM). *: significant increase in *G* (p<0.005). The effect of *Dermatophagoides pteronyssinus* (20 µg·mL⁻¹ *Der pI*) + DTT (1.5 mM) was different from that of other variables (p<0.001).

Discussion

This study shows that *D. pteronyssinus* allergens increase epithelial permeability, as assessed by airway conductance (*G*). This effect is probably due to the cysteine protease enzymatic activity of the allergen, since it was observed only in the presence of DTT, an activator of cysteine proteases, and it was inhibited by E-64, an inhibitor of cysteine proteases which has no effect on *D. pteronyssinus* serine proteases [10, 16]. *D. pteronyssinus*-induced increase in *G* could be entirely attributed to an increase in the paracellular conductance. Finally, *D. pteronyssinus* allergens have no significant effect on active electrogenic ion transports, as assessed by *I*_{sc}. Changes in epithelial paracellular conductance were not associated with histologic evidence of epithelial injury or electrophysiological evidence of rupture in epithelial continuity. These data suggest that the cysteine protease activity of *D. pteronyssinus* affects epithelial paracellular pathways at doses lower than those required to induce cell detachment or cell death.

The magnitude of the *D. pteronyssinus*-induced increase in airway epithelial conductance was small, which prevents any conclusion as to whether it may be associated with an increase in epithelial permeability to proteins to be drawn; this small magnitude may be explained by the deliberately smaller concentrations of allergens to which the tracheal fragments are exposed, compared to those that induced cell detachment and/or increased epithelial permeability in other studies [8, 10–12]. The physiological and clinical relevance of such a small effect may be questioned. However, some sites of the airways may be exposed to much higher concentrations of allergens, since each *D. pteronyssinus* faecal pellet contains up to 0.1 ng *Der pI* in a sphere

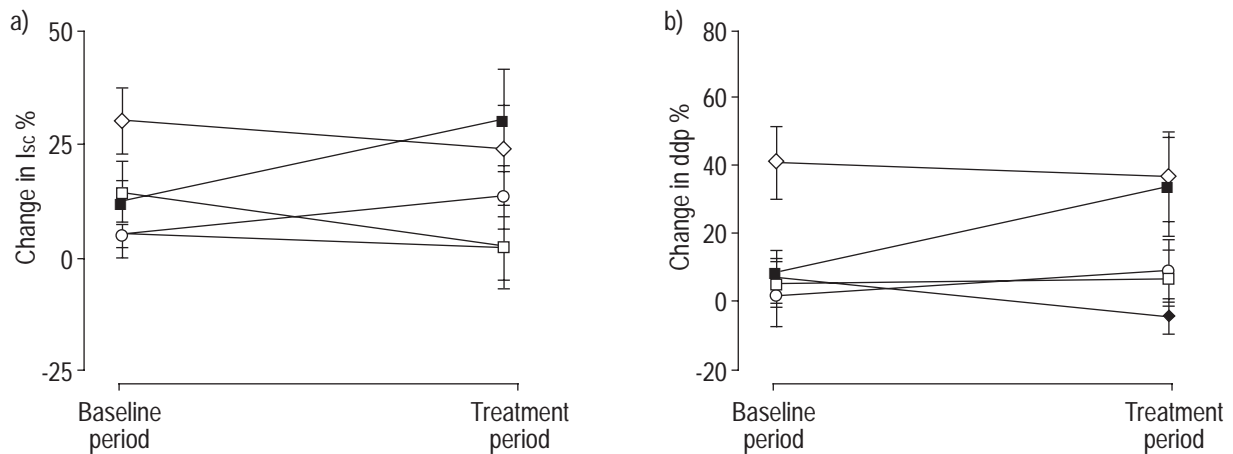


Fig. 2. – Change in a) short-circuit current (I_{sc} ; and b) potential difference (PD), during the 30-min baseline period and the 30-min test period. Data are expressed as % of the initial baseline value. $n=12$ for each condition. *D. pteronyssinus*: *Dermatophagoides pteronyssinus*; (○): *D. pteronyssinus* ($20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI*) + DTT (1.5 mM); (■): *D. pteronyssinus* ($2 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI*) + DTT (1.5 mM); (◆): *D. pteronyssinus* ($20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI*) without DTT; (◇): *D. pteronyssinus* ($20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI*) + DTT (1.5 mM) in the presence of E64 (10 nM); (□): control, *i.e.* mite growth medium + DTT (1.5 mM). *: tested agents induced no significant effect.

of $20 \mu\text{m}$ in diameter, which corresponds to a concentration of $\sim 10 \text{mg}\cdot\text{mL}^{-1}$ in the pellet [17, 18]. Moreover, experimental conditions only allowed determination of the consequences of a single, and brief, *in vitro* exposure. Since the increase in airway epithelial conductance after exposure to $20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI* + DTT was a slow process, the effect of repeated and/or prolonged exposure may be greater.

The cysteine protease-induced increase in epithelial paracellular conductance could be the consequence of: direct alteration of intercellular junctions by the cysteine-protease activity of *Der pI*; or inflammatory phenomena, either induced by the cysteine protease of *D. pteronyssinus*, or that lead to the production of cysteine proteases by inflammatory cells. Such phenomena could be mediated by *D. pteronyssinus*-induced release of cytokines such as monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-6, or IL-8, which production by epithelial cells is enhanced by house dust mite proteases [11]; other molecules such as soluble CD23 (sCD23) may also play a role, since *Der pI* induces the release of this product by cleavage of membrane-bound CD23, which in turn increases the production of IgE by B-cells [19]. Finally, other inflammatory mediators may be involved, since *Der pI* activates the pro-inflammatory transcriptional factor NF- κ B [20]. However, the effect of such mediators and cytokines on airway epithelial bioelectric properties is unknown. Furthermore, a role of inflammatory cells is unlikely, since Lewis rats were not previously sensitized to *D. pteronyssinus* and were free of respiratory diseases, and since histologic scores of mucosal infiltration by inflammatory cells were similar in fragments exposed to *D. pteronyssinus* ($20 \mu\text{g}\cdot\text{mL}^{-1}$) + DTT and in fragments exposed to the control solution.

Thus, a direct enzymatic effect on intercellular junctions appears to be the most likely explanation for the *D. pteronyssinus*-induced increase in airway epithelial paracellular conductance. Tight junctions are made of proteins [12, 21–23], and are known to be damaged by exogenous enzymes

such as elastase from *Pseudomonas aeruginosa* [24]. Cysteine protease and serine protease fractions of *D. pteronyssinus* have also been shown to damage tight junctions of epithelial cell monolayers [10, 12]; this effect appears to be due to a cleavage of at least two tight junction proteins, occludin and claudin-1, in which some putative cleavage sites have been identified [12]. In our study, *D. pteronyssinus* serine-proteases did not appear to participate in the effect of the crude *D. pteronyssinus* extract on airway epithelial paracellular conductance, since this effect was observed only in the presence of DTT, an activator of cysteine proteases, and was entirely inhibited by E-64, which does not affect the activity of *D. pteronyssinus* serine proteases [10, 16]. An additional minor effect of serine proteases can not be completely ruled out since the effect of serine protease activators and inhibitors was not studied. However, WAN *et al.* [12] found no effect

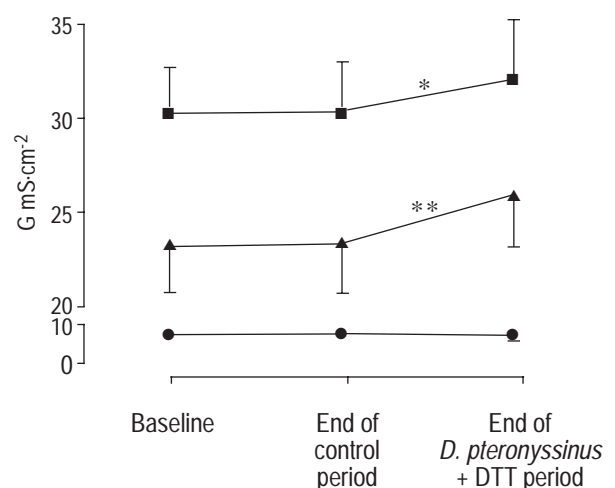


Fig. 3. – Effect of control solution (mite growth medium + 1.5 mM DTT) and *D. pteronyssinus* ($20 \mu\text{g}\cdot\text{mL}^{-1}$ *Der pI*) with DTT (1.5 mM) on total (G, ■), paracellular (G_{para} , ▲) and transcellular (G_{cell} , ●) conductances in eight tracheal fragments **: $p<0.01$; *: $p<0.05$.

of serine protease inhibitors on the effect of *D. pteronyssinus* on tight junctions in epithelial monolayers of human origin.

As in studies by HERBERT *et al.* [8] and TOMEE *et al.* [11], evidence of cell death, which would have dramatically affected the bioelectric variables in our experiments was not observed. Conversely, WINTON *et al.* [10] found that the increase in the paracellular conductance of epithelial cell monolayers was associated with initiation of cell death. These discrepancies may be due to a greater susceptibility of the cell lines they used to protease-induced apoptosis. An alternative explanation would be that the protease activities, which induced apoptosis of epithelial cells in their study, correspond to very high concentrations of allergens.

In the studies by HERBERT *et al.* [8] and TOMEE *et al.* [11], concentrations of *Der pI* similar to ours (*i.e.* 2–20 $\mu\text{g}\cdot\text{mL}^{-1}$) induced a significant cell detachment only in cultured cells, while much higher concentrations (*i.e.* 300 $\mu\text{g}\cdot\text{mL}^{-1}$) were required to increase epithelial permeability to albumin and detach epithelial cells in isolated bronchial segments; this is probably due to differences in cohesiveness between cultured epithelium and tissues, and may explain why cell detachment in tracheal tissues with *Der pI* concentrations of 20 $\mu\text{g}\cdot\text{mL}^{-1}$ was not observed. Therefore, comparisons between studies must be cautious since epithelial permeability and cohesiveness may be different from one species (or cell line) to another.

HERBERT *et al.* [8] observed that *Der pI* induced a detachment of MDCK cells even without addition of reducing agents, which suggests that these cells (or their culture medium) were able to reduce the cysteine protease by themselves. This was not the case in the present study, which could be related to a lower thiol-reducing potential of rat tracheal cells compared with MDCK cells. Similarly, the lack of effect of the cysteine protease activity that was reported by TOMEE *et al.* [11] may be due to a low thiol-reducing potential of the cell lines used or its culture medium. In humans, airway secretions contain agents such as glutathione, which can activate cysteine-proteases [8].

The *in vivo* relevance of doses of allergen used in *in vitro* studies is difficult to assess. During usual domestic activity, doses of allergens, to which subjects are environmentally exposed, are very low, although they are likely to increase during close contact with reservoirs such as mattresses [17, 18]. Besides, although eighty percent of mite allergens are contained in particles which have a diameter of more than 10 μm [17, 25], up to 15% of such particles can deposit in the human airways [26], so that an individual may inhale >200 mite faecal pellets per day [12]. Considering that each faecal particle contains ~10 $\text{mg}\cdot\text{mL}^{-1}$ *Der pI* [17], it may be hypothesized that very high local concentrations are reached in some limited areas of the airways when allergens elute from these particles in airway lining fluid [12, 18].

In conclusion, the use of electrophysiological techniques allowed to demonstrate that the cysteine protease activity of house dust mite allergens increases the paracellular epithelial permeability of airway tissues at concentrations lower than those which induce cell detachment or cell death. If such a phenomenon is relevant *in vivo*, the resulting increase in epithelial permeability may facilitate the penetration of allergens and toxic agents in the airway

mucosa and, thereby, the contact between these agents and inflammatory cells. It may also enhance the transepithelial passage of inflammatory mediators. Thus, the enzymatic properties of *D. pteronyssinus* may play a role in the initiation and amplification of the bronchial immunological and inflammatory phenomena that are observed in asthma.

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