

House dust mite allergen exerts no direct proinflammatory effects on human keratinocytes

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Background: *Dermatophagoides pteronyssinus* is a trigger of atopic dermatitis. Many *D pteronyssinus* allergens are proteases that can elicit airway inflammation by stimulating the release of cytokines and chemokines by bronchial epithelial cells.

Objective: We sought to investigate whether *D pteronyssinus* allergens can exert a similar activity on skin keratinocytes.

Methods: Primary cultures of keratinocytes from healthy subjects or patients with atopic dermatitis and normal human bronchial epithelial cells were compared for cytokine production in response to *D pteronyssinus* extract.

Results: Keratinocytes, but not bronchial epithelial cells, displayed a modest dose-dependent release of IL-1 α and IL-1 receptor antagonist but no induction of their mRNA after exposure to *D pteronyssinus*. However, *D pteronyssinus* also degraded these cytokines. On the other hand, *D pteronyssinus* extract induced bronchial epithelial cells, but not keratinocytes, to increased expression of IL-8/CXCL8 and GM-CSF mRNA and protein. These effects were efficiently abrogated by a mixture of cysteine and serine protease inhibitors. Both IL-8 and GM-CSF were fully resistant to *D pteronyssinus* proteolytic attack. No induction of monocyte chemoattractant protein 1/CCL2, RANTES/CCL5, or IFN- γ -induced protein of 10 kd/CXCL10 was detected in either cell type. Only bronchial epithelial cells expressed protease-activated receptor (PAR) 4 mRNA, whereas PAR-1, PAR-2, and PAR-3 mRNA was found in both cell types. *D pteronyssinus* did not affect PAR mRNA signals.

Conclusions: Although *D pteronyssinus* can cause proteolysis-dependent release of cytokines from keratinocytes, it appears incapable of activating de novo expression of cytokines and chemokines, arguing against a direct proinflammatory activity of house dust mite on the skin. (J Allergy Clin Immunol 2002;109:532-8.)

Key words: Epithelial cells, *Dermatophagoides pteronyssinus*, cytokines, chemokines, atopic dermatitis

The house dust mite *Dermatophagoides pteronyssinus* is a major source of aeroallergens implicated in the expression of atopic disorders, including asthma, allergic rhinitis, and atopic dermatitis (AD). In particular, strong circumstantial evidence suggests that *D pteronyssinus* antigens are important precipitating factors of AD.^{1,2} Randomized control trials have demonstrated that extreme reduction in house dust mite levels improves the clinical manifestations of this disease.^{3,4} Moreover, the epicutaneous application of *D pteronyssinus* extracts in patients with AD induces skin lesions resembling the naturally occurring eczema,^{5,6} and significant numbers of specific T lymphocytes can be isolated from positive patch test reactions.⁷ Most patients with AD reacting to a *D pteronyssinus* atopy patch test show high levels of specific IgE in the serum.^{2,8} However, patch test reactions can be elicited also in patients with no specific IgE, raising the possibility that non-IgE-mediated immune mechanisms may be involved or that *D pteronyssinus* may directly cause inflammatory changes in the skin.^{2,8,9}

The detailed characterization of *D pteronyssinus* extracts has shown that at least 4 of the 9 distinct groups of allergens that have been purified express proteinase activity. The group 1 allergens are cysteine proteinases, whereas the group 3, 6, and 9 allergens share sequence identity and enzymatic activity profiles with archetypal serine proteinases.¹⁰ Respiratory epithelial cells are recognized as a primary target of *D pteronyssinus* proteolytic activity. Experiments conducted on bronchial epithelial cell monolayers suggest that Der p 1 increases paracellular permeability through a noncytotoxic disruption of intercellular tight junctions, hence potentiating the availability of environmental aeroallergens to dendritic antigen-presenting cells.^{11,12} Moreover, total *D pteronyssinus* extract and its purified allergens Der p 1, Der p 3, and Der p 9 can activate the proinflammatory functions of airway epithelial cells, leading to the production of cytokines and chemokines implicated in the local inflammatory response.¹³⁻¹⁵

A role for *D pteronyssinus* allergens as activators of keratinocytes appears as an intriguing possibility also in the pathogenesis of AD. Although *D pteronyssinus* components have high molecular weight that prevents their penetration into intact normal skin, the epidermal barrier perturbations characterizing AD may favor the invasion

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Abbreviations used

AD:	Atopic dermatitis
ICAM-1:	Intercellular adhesion molecule 1
IL-1ra:	IL-1 receptor antagonist
IP-10:	IFN- γ -induced protein of 10 kd
MCP-1:	Monocyte chemoattractant protein 1
PAR:	Protease-activated receptor

of *D pteronyssinus* proteins into the lower epidermal compartment. This study was thus undertaken to determine whether *D pteronyssinus* extracts could promote the production, release, or both of proinflammatory mediators by human keratinocytes compared with bronchial epithelial cells. In particular, we have examined the release of the cytokines IL-1 α , IL-1 receptor antagonist (IL-1ra), and GM-CSF and of the chemokines IL-8/CXCL8, RANTES/CCL5, monocyte chemoattractant protein 1 (MCP-1/CCL2), and IFN- γ -induced protein of 10 kd (IP-10/CXCL10). These substances are generated by skin and respiratory epithelial cells during AD and bronchial asthma, respectively, and are implicated in the pathophysiology of atopic inflammation.¹⁶⁻¹⁹

METHODS

Cell cultures

Primary cultures of human keratinocytes were prepared from plastic surgery skin obtained from 4 healthy nonatopic subjects (3 women, 25-38 years of age; 1 man, 41 years of age) and from normal-appearing skin of the forearms of 4 patients with definite AD diagnosed according to standard criteria (2 women, 22 and 34 years of age; 2 men, 25 and 43 years of age), as previously described.¹⁶ One patient had pure AD with no elevated serum IgE levels, whereas 3 patients also had respiratory atopy and had elevated serum IgE levels (345-31,000 kU/mL). Informed consent was obtained from all subjects, and the study was approved by the local ethical committee. Keratinocytes were cultured in the serum-free medium Keratinocyte Growth Medium (Clonetics, San Diego, Calif). Primary normal human bronchial epithelial cells and Bronchial Epithelial cell Growth Medium were obtained from Clonetics. Hydrocortisone, retinoic acid, and epinephrine, which are known to affect cytokine-chemokine expression in epithelial cells,^{16,20} were removed from the media 24 hours before experiments.

Reagents

Freeze-dried extract was obtained from the medium of a crude *D pteronyssinus* culture (Stallergènes, Saronno, Italy). To recover the whole allergenic content,²¹ ammonium bicarbonate was added gradually to the stirred supernatant at 4°C to achieve 80% saturated solution. The pellet was resuspended in distilled water, dialyzed, and finally filter sterilized. In some experiments total *D pteronyssinus* extract from Kallergen (Settimo Milanese, Italy) was used, with identical results. Total grass pollen extract was provided by Stallergènes. Preparation of *D pteronyssinus* extract is known to deactivate intrinsic cysteine protease activity caused by cysteine oxidation.^{10,22} Thus a 15-minute preactivation of *D pteronyssinus* with 5 mmol/L cysteine HCl (Sigma-Aldrich, Milan, Italy), pH 7.0, at 37°C was always performed before use. The protease inhibitors E-64 and aprotinin were purchased from Sigma-Aldrich.

Casein-Coomassie blue assay for proteolytic activity of *D pteronyssinus*

The assay was performed according to a standardized protocol,²³ with minor modifications. Briefly, 2 mg/mL casein (Sigma-Aldrich) and increasing doses of papain (from Papaya latex, Sigma code P3125) or *D pteronyssinus* extracts were dissolved in 5 mmol/L cysteine and 50 mmol/L Tris-HCl (pH 7.0) and then incubated for 30 minutes at 25°C with or without protease inhibitors. Thereafter, 25 μ L of the assay mixture was diluted in 775 μ L of distilled water and then supplemented with 200 μ L of Bio-Rad protein dye reagent concentrate, containing 0.05% (wt/vol) Coomassie brilliant blue G-250 (Bio-Rad Labs, Munich, Germany). After vortexing, samples were read at 595 nm. Results are expressed as the percentage of residual casein.

RNA analysis

Total RNA was extracted with a modified guanidine isothiocyanate-acid phenol protocol, as previously described.¹⁶ RNase protection assay of IL-1 α , IL-1 β , and IL-1ra was performed with a commercial multiprobe template set (hCK-2) from BD Pharmingen (San Diego, Calif). For simultaneous analysis of GM-CSF, RANTES, MCP-1, IP-10, and IL-8, a template set was prepared by BD Pharmingen, with the housekeeping gene *L32* used as an internal control. The primers and PCR details for the amplification of protease-activated receptor (PAR) 1 (708 bp), PAR-2 (582 bp), PAR-3 (382 bp), and PAR-4 (392 bp) were taken from Wan et al.¹² A glyceraldehyde-3-phosphate dehydrogenase primer set was used as an internal control. For semiquantitative analysis, RNA concentrations, primers, and PCR cycles were titrated to obtain standard curves for verification of linearity. RNase protection assay and PCR bands were quantified by using laser densitometry supported by the Quantity One software (Bio-Rad, Hercules, Calif), with each signal normalized to the corresponding internal control band.

ELISA

Cytokines released by cell cultures were measured on cell-free supernatants harvested after 3 to 24 hours of treatment. IL-1 α , IL-1 β , and IL-1ra were determined with Quantikine ELISA kits from R&D Systems (Abingdon, Oxon, United Kingdom). GM-CSF and IL-8 were measured with OptEIA ELISA kits (BD Pharmingen). Data are expressed as picograms or nanograms per 10⁶ cells $\pm$ SD. ELISA kits were also used to evaluate *D pteronyssinus*-induced degradation of recombinant human IL-1 α , IL-1ra, GM-CSF, or IL-8 (R&D Systems). Data are expressed as the percentage of residual cytokines $\pm$ SD.

Statistical analysis

The Wilcoxon signed-rank test was used to compare differences in cytokine release. Significance was assumed at a *P* value of .05 or less.

RESULTS

D pteronyssinus proteolytic activity

To assess the proteolytic activity of *D pteronyssinus* extracts, we used the casein-Coomassie blue assay. *D pteronyssinus* extract induced a dose-dependent degradation of casein with 20-fold lower potency than the pure cysteine enzyme papain, so that 10 μ g/mL *D pteronyssinus* extract and 0.5 μ g/mL papain resulted in the loss of 15% of the initial substrate (Fig 1). The use of the cysteine protease-specific inhibitor E-64 alone and together with the serine protease inhibitor aprotinin allowed us to establish that 85% of total *D pteronyssinus* caseinolytic activity was due to these 2 classes of enzymes, with ser-

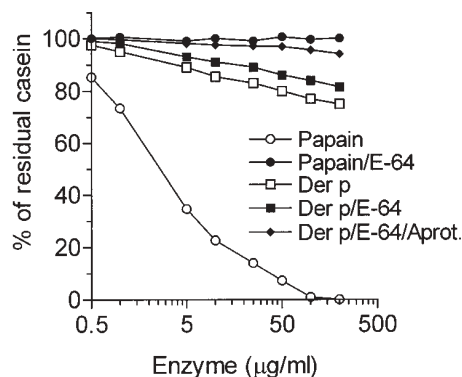


FIG 1. *D pteronyssinus* (Der p) extract contains serine and cysteine proteolytic activities. Increasing concentrations of enzyme were incubated with a fixed dose of substrate (2 mg/mL casein) for 30 minutes at 25°C. Residual substrate concentration was measured by using the casein-Coomassie blue assay. Identical results were obtained in different experiments ($n = 6$). Aprot, aprotinin.

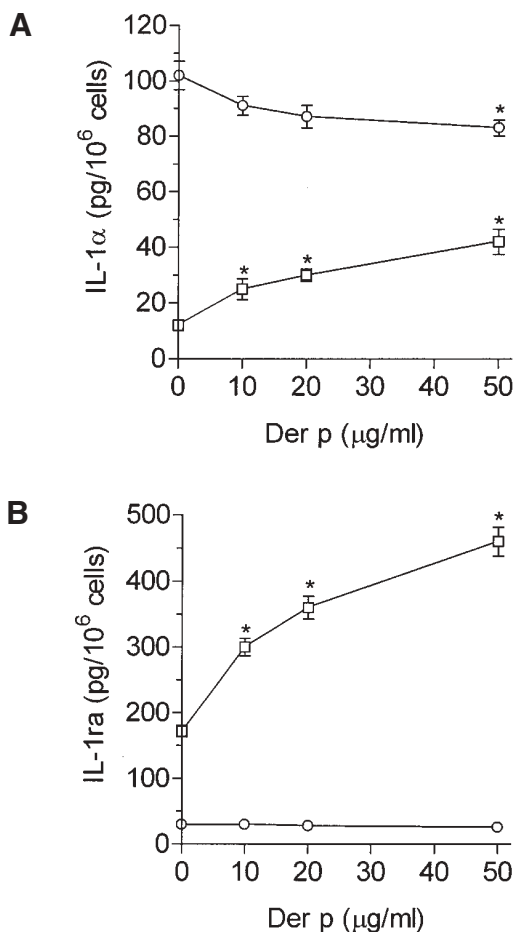


FIG 2. *D pteronyssinus* (Der p) extract determines a dose-dependent release of both IL-1 α (A) and IL-1ra (B) from keratinocytes but not from bronchial epithelial cells. Results are from ELISA on supernatants of keratinocytes from healthy donors ($n = 4$, squares) and bronchial epithelial cells ($n = 4$, circles) incubated with increasing doses of *D pteronyssinus* extract for 6 hours. * $P < .05$ versus untreated cultures.

ine proteases accounting for most of the *D pteronyssinus* proteolytic activity. Only about 5% of total *D pteronyssinus* activity was due to cysteine proteases.

Cytokine release and expression

Keratinocytes constitutively express high levels of preformed IL-1 α , IL-1 β , and their antagonist IL-1ra, and the release of these cytokines is considered an early signal of cell membrane perturbation. Keratinocytes and bronchial epithelial cells differed in their relative proportion of IL- α and IL-1ra basal release. Keratinocytes secreted much higher levels of IL-1ra than IL-1 α , with an IL-1ra/IL-1 α ratio of 10 to 15, as previously reported.¹⁷ In contrast, bronchial epithelial cells released about 2-fold higher levels of IL-1 α than IL-1ra, with an IL-1ra/IL-1 α ratio of 0.4 to 0.6 (Fig 2). *D pteronyssinus* extract caused a dose-dependent and parallel increase of both IL-1 α and IL-1ra in the keratinocyte supernatant (Fig 2), whereas a reduction of IL-1 α and unchanged IL-1ra levels were detected in medium from bronchial epithelial cells. Time-course experiments showed that *D pteronyssinus* induced a maximal IL-1 α and IL-1ra increase in keratinocyte supernatants at 6 hours (Fig 3, A and C). IL-1 α returned to basal levels after 9 hours' treatment with *D pteronyssinus* and then increased again at 24 hours, whereas IL-1ra maintained high but approximately constant levels. The culture medium from bronchial epithelial cells showed a significant decrease in the levels of IL-1 α at 6 to 9 hours and of IL-1ra at 24 hours (Fig 3, B and D). Use of specific enzyme inhibitors abrogated these effects almost completely, indicating that they were related to *D pteronyssinus* proteolytic activity. Of note, neither *D pteronyssinus* nor enzyme inhibitors caused any change in cell viability and cell-to-cell or plastic adherence (data not shown). IL-1 β could not be detected in the supernatants of either cell type. The activity of *D pteronyssinus* was similar on keratinocytes cultured from patients with AD and healthy donors, although the former secreted basally higher levels of both IL-1 α and IL-1ra, as described previously.¹⁷

D pteronyssinus-induced variations of IL-1ra and, especially, IL-1 α levels in keratinocyte medium were suggestive of an early cytokine release, followed by degradation, whereas this last process appeared prevalent in bronchial epithelial cell cultures. Indeed, *D pteronyssinus* extract dose-dependently degraded recombinant IL-1 α and, to a lesser extent, IL-1ra (Fig 4), a phenomenon that could be prevented by a mixture of E-64 and aprotinin. An RNase protection assay was performed to see whether *D pteronyssinus* affected cytokine expression. No perturbation of IL-1 α or IL-1ra mRNA basal levels could be observed in either keratinocytes or bronchial epithelial cells after 3 to 24 hours' contact with *D pteronyssinus* extract (not shown). In addition, *D pteronyssinus* did not affect GM-CSF and IL-8 neosynthesis by either AD or normal keratinocytes (Fig 5). In contrast, *D pteronyssinus* elicited a weak, transient induction of GM-CSF mRNA, perceivable at 3 and 24 hours, with a 2-fold increase over background levels and

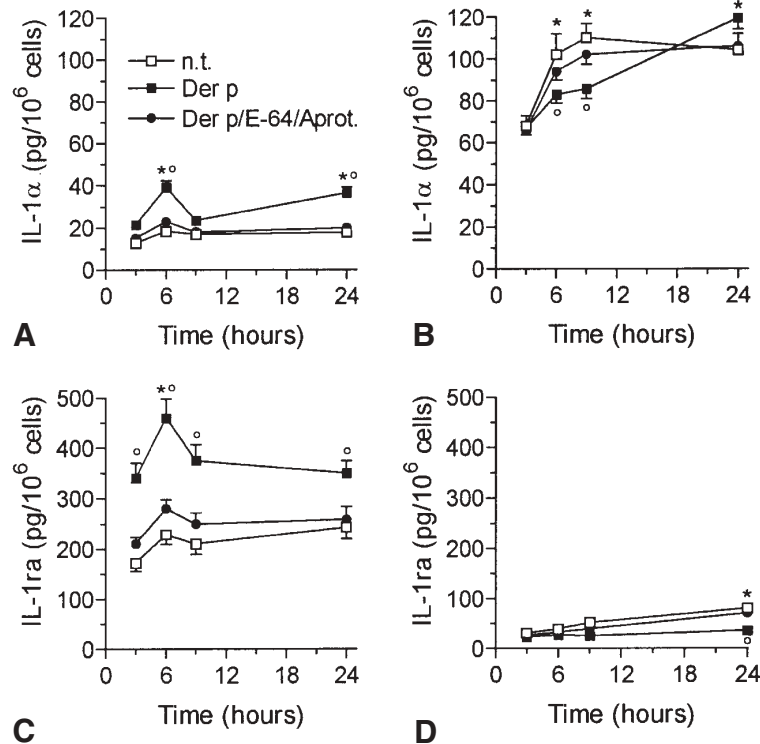


FIG 3. *D pteronyssinus* (*Der p*) determines early release of IL-1 α and IL-1ra in the supernatant of keratinocytes. Results are from ELISA on supernatants of keratinocytes from healthy donors ($n = 4$, **A** and **C**) and bronchial epithelial cells ($n = 4$, **B** and **D**) treated with 50 μ g/mL *D pteronyssinus* extract for 3 to 24 hours. For neutralization of serine and cysteine *D pteronyssinus* proteases, both aprotinin (*Aprot*) and E-64 were added at 50 μ g/mL. * $P < .05$ versus cytokine release at 3 hours' stimulation with *D pteronyssinus*. ° $P < .05$ versus untreated cultures.

an 8- to 10-fold increase in IL-8 mRNA in bronchial epithelial cells at 24 hours (Fig 5). GM-CSF and IL-8 mRNA induction by *D pteronyssinus* was dependent on its proteolytic activity. In keeping with experiments conducted with purified *D pteronyssinus* proteases¹⁴ and with our mRNA data, we also observed a significant accumulation of both GM-CSF and IL-8 protein at 24 hours only in bronchial epithelial cells that was again dependent on preservation of cysteine-serine protease activity (Fig 6). *D pteronyssinus* extract did not cause any degradation of the corresponding recombinant molecules (Fig 4). As a further control, keratinocytes and bronchial epithelial cells were also stimulated with total grass pollen extract, but no induction of cytokines or chemokines was detected. *D pteronyssinus* did not induce RANTES, IP-10, or MCP-1 mRNA or protein. Finally, no membrane intercellular adhesion molecule 1 (ICAM-1) expression could be observed in either cell type after stimulation with *D pteronyssinus* extracts, whereas they could partially remove membrane ICAM-1 induced by IFN- γ (data not shown).

PAR expression

Induction of cytokine expression by *D pteronyssinus* serine proteases in bronchial epithelial cells could be mediated in part by activation of a new family of G protein-coupled receptors, the PARs.^{14,15} To verify whether

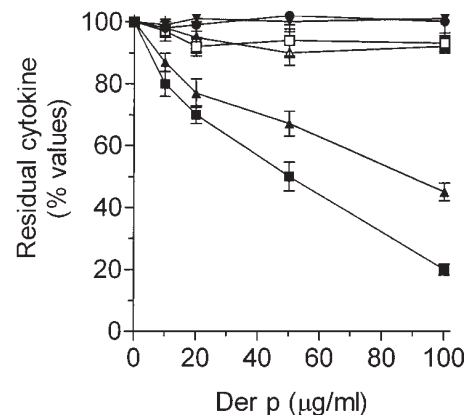


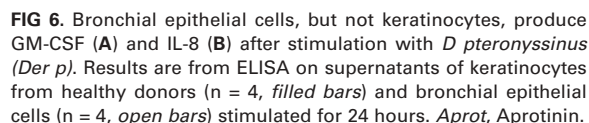
FIG 4. *D pteronyssinus* (*Der p*) extract determines a dose-dependent proteolytic degradation of recombinant human IL-1 α and IL-1ra. A dose of 500 pg/mL IL-1 α (filled squares), IL-1ra (filled triangles), GM-CSF (filled inverted triangles), or IL-8 (filled circles) was incubated with increasing concentrations of *D pteronyssinus* extract for 6 hours at 37°C ($n = 6$). Combined addition of aprotinin and E-64 (both at 50 μ g/mL) prevented degradation of IL-1 α (open squares) and IL-1ra (open triangles).

a distinct pattern of PAR expression could underlie the different responsiveness of keratinocytes and bronchial epithelial cells, we performed semiquantitative RT-PCR analysis of the 4 characterized PARs. By means of densitometric analysis, we could not find relevant differ-



Increasing evidence indicates that several proteases of the house dust mite *D pteronyssinus* have a direct proinflammatory role in the respiratory tract. These enzymes can alter the integrity of the airway barrier and thus favor allergen penetration and capture by dendritic antigen-presenting cells.^{11,12,21} Furthermore, *D pteronyssinus* proteases have been proposed as possible initiators of local inflammation by promoting the release of potent cytokines and chemokines by the airway epithelium.¹³⁻¹⁵ *D pteronyssinus* allergens can also trespass the stratum corneum and penetrate into the deeper epidermal layers,^{24,25} a phenomenon favored by conditions that can be easily observed in patients with AD, such as scratched and dry skin or the presence of ongoing inflammation.²⁶ Keratinocytes are critically involved in the initiation and amplification of inflammatory and immune responses, including those associated with AD.^{16-18,27} Hence we hypothesized that *D pteronyssinus* proteases could be among the exogenous stimuli reasonably implicated in the triggering of keratinocyte activation. In agreement with previous characterizations of *D pteronyssinus* extracts, our preparations displayed a predominant serine proteolytic activity and a minor contribution of cysteine proteases.^{10,21,28}

In the present study we have shown that human keratinocytes and bronchial epithelial cells differed substantially in their spontaneous, as well as their *D pteronyssinus*-induced, IL-1 α and IL-1ra release. Keratinocytes



appeared protected from IL-1 α bioactivity by the highly predominant release of IL-1ra. In contrast, bronchial epithelial cells released high levels of IL-1 α and low levels of IL-1ra and were thus set up to an easier proinflammatory behavior. In response to a variety of activation signals, IL-1 α is overproduced compared with IL-1ra¹⁷ and determines the autocrine induction of itself and other mediators, including IL-8 and GM-CSF.²⁹ An early accumulation of both IL-1 α and IL-1ra could be clearly induced in keratinocytes by *D pteronyssinus* in a serine-cysteine protease-dependent fashion but only with modest reduction of the IL-1ra/IL-1 α ratio (from 10-15 to 8-10). *D pteronyssinus* did not unbalance significantly the IL-1 axis in bronchial epithelial cells, and the proteolytic degradation of spontaneously released IL-1 α appeared as the predominant phenomenon. *D pteronyssinus* did not induce neosynthesis of IL-8 and GM-CSF in keratinocytes from either healthy subjects or patients with AD. It is reasonable to posit that the reduced control over IL-1 α bioactivity found in bronchial epithelial cells is responsible for the pronounced basal production of IL-8 and GM-CSF. Moreover, bronchial epithelial cells displayed an early induction of GM-CSF and IL-8 mRNA in response to *D pteronyssinus*, which could be efficiently neutralized by a mixture of serine and cysteine protease inhibitors. The amount of IL-8 released in the medium in response to 50 μ g/mL total *D pteronyssinus* extract was quantitatively similar to that provoked by 0.1 μ g/mL purified cysteine protease Der p 1 or serine protease Der p 9, whereas GM-CSF release was 10-fold lower in response to total *D pteronyssinus* extract.¹⁴ Such marked differences possibly suggest that the active components are highly diluted in the total extract or that its complex composition includes protease inhibitors that attenuate the final activity of *D pteronyssinus* proteases on responding cells.

Relevant to the action of serine peptidase allergens in epithelial cells is the G protein-linked family of PAR receptors.^{30,31} In particular, the PAR-2 receptor has been suggested to mediate Der p 3 and Der p 9 activity on bronchial epithelial cell lines.¹⁵ However, the distinct sensitivity of keratinocytes and bronchial epithelial cells to *D pteronyssinus* extracts cannot be attributed to a differential expression of this receptor because high levels of functional PAR-2 have been described in both cell types.^{32,33} We observed that keratinocytes and bronchial epithelial cells expressed similar amounts of the specific transcripts of PAR-1, PAR-2, and PAR-3, whereas PAR-4 mRNA was detected only in bronchial epithelial cells. Enzyme-induced cleavage of PARs leads to their endocytosis and degradation in lysosomes and to a parallel rapid neosynthesis.³¹ The lack of de novo transcription of PAR genes, either in keratinocytes or bronchial epithelial cells, after 6 hours' stimulation with *D pteronyssinus* extract suggests that *D pteronyssinus* activation of bronchial epithelial cells may follow a PAR-independent mechanism.

Our experiments demonstrated that *D pteronyssinus* extract did not possess the intrinsic capacity to elicit ker-

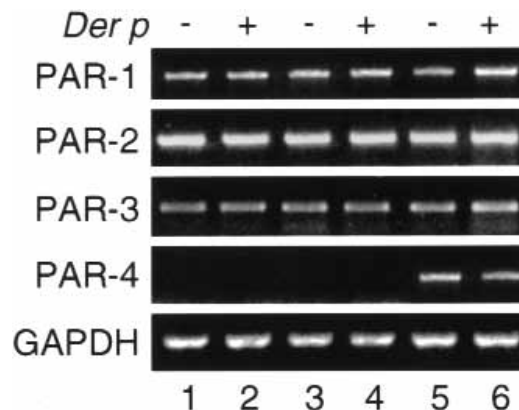


FIG 7. Expression of PARs in keratinocytes and bronchial epithelial cells. Semiquantitative RT-PCR analysis was performed on 1 μ g of total RNA extracted from untreated cells or after treatment with 50 μ g/mL *D pteronyssinus* (Der p) for 6 hours. Lanes 1 and 2, healthy keratinocytes; lanes 3 and 4, AD keratinocytes; lanes 5 and 6, bronchial epithelial cells. Similar results were confirmed in 4 independent experiments. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

atinocyte proinflammatory functions in terms of increased expression of cytokines, chemokines, and ICAM-1 in vitro. We have previously observed that keratinocytes cultured from patients with AD tend to spontaneously release higher amounts of proinflammatory mediators, including IL-1 α , IL-1ra, GM-CSF, and RANTES, when compared with keratinocytes from healthy control subjects. Furthermore, they respond to a variety of physiologic stimuli with an earlier and exaggerated expression of these molecules.^{16-18,27} In contrast, *D pteronyssinus* extracts could not induce proinflammatory activation of keratinocytes from patients with AD. Our results argue against the possibility that *D pteronyssinus* proteases exert a direct proinflammatory activity on keratinocytes and suggest that the ability of *D pteronyssinus* extracts to trigger AD and atopy patch test responses is largely mediated by specific immune mechanisms.³⁴ However, we cannot exclude that keratinocytes could provide a different response in vivo, especially under conditions of ongoing inflammation in which keratinocytes are exposed to multiple activation signals. Moreover, it is still possible that *D pteronyssinus* can affect other epidermal cell types that in turn could modulate keratinocyte inflammatory functions.

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