Immunomodulation of Skin Cytokine Secretion by House Dust Mite Extracts

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Key Words

Immunomodulation · House dust mite · Human skin equivalent · Cytokines · Chemokines

Abstract

Background: Skin contact with house dust mites may contribute to atopic dermatitis and other skin diseases. We sought to determine if molecules from house dust mites could influence the release of proinflammatory cytokines and chemokines from epidermal keratinocytes and dermal fibroblasts grown in a human skin equivalent (HSE) model.

Methods: HSEs consisting of an epidermis of keratinocytes with stratum corneum over a dermis of fibroblasts in a collagen matrix were challenged with *Dermatophagoides farinae*, *D. pteronyssinus* and *Euroglyphus maynei* mite extracts.

Results: HSEs secreted interleukin (IL)-1 α , IL-1 receptor antagonist, IL-6, IL-8, cutaneous T cell-attracting chemokine, transforming growth factor- α , granulocyte/macrophage and macrophage colony-stimulating factors and vascular endothelial cell growth factor in response to at least 1 mite extract. Extracts of different mite species stimulated HSEs to release different cytokines. Therefore, extracts of different species contained different molecules or different concentrations of similar molecules. The cytokine release profiles of cells in the HSEs were not the same as for monocultured keratinocytes and fibroblasts. **Conclusions:** Molecules

from house dust mites are capable of inducing the release of multiple proinflammatory cytokines and chemokines from epidermal keratinocytes and dermal fibroblasts. Avoiding skin contact with house dust mites would reduce the possibility of mite-induced inflammation in the skin. Therefore, measures to reduce contact with mite molecules such as frequent vacuuming of upholstered furniture and carpets and laundering of clothing and bedding to remove mite molecules and allergens could reduce skin contact with mite molecules and diminish exacerbations of skin inflammation in patients with atopic dermatitis and other skin diseases.

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Introduction

The house dust mites *Dermatophagoides farinae*, *D. pteronyssinus* and *Euroglyphus maynei* are ubiquitous inhabitants of homes worldwide and are particularly prevalent in homes in humid geographical areas. Within homes they are most abundant in high-use areas where skin scales accumulate and serve as their food. This includes carpets, upholstered furniture such as couches, recliners and chairs, and mattresses and bedding. Human skin may come into contact with dust mites when a person uses furniture and beds or engages in activities on car-

pets. There are reports of house dust mites being recovered from human skin and clothing [1–4].

A defective skin barrier may allow penetration of mite molecules into the lower epidermis and dermis [5–10]. Molecules in mite material include both allergens and nonallergens, and some of these are enzymes. House dust mite group 1 allergens are cysteine proteases, and groups 3, 6 and 9 are serine proteases [11]. Protease enzymes from house dust mites, solvents, volatile organics, soaps, detergents, surfactants and abrasive materials can damage the skin epidermal surface barrier, and this can allow penetration of mite molecules into the lower epidermis and dermis [6, 12]. The recombinant allergen Der f 1 is a cysteine protease that has been shown to reduce the barrier function of skin in nude mice [13]. Positive patch test reactions to house dust mites have been reported [14–16]. Serine peptidases from fecal pellets of *D. pteronyssinus* cleave occludin of epithelial tight junctions of cultured airway epithelial cells [17, 18]. Likewise, *D. farinae* and *D. pteronyssinus* extracts cause changes in growth and reduced adhesion in cA549 type II epithelial cells [19].

This chemical and physical disruption may allow penetration of molecules that may induce epidermal and dermal inflammation and immune responses that are mediated by cytokines and chemokines from stimulated keratinocytes, fibroblasts, microvascular endothelial cells and infiltrating cells, including Langerhans cells, lymphocytes and monocytes/macrophages, neutrophils and eosinophils. Stimulated keratinocytes and fibroblasts produce and secrete multiple cytokines that promote cutaneous inflammation. Previous research has shown that house dust mite and stored product mite extracts stimulate the secretion of cytokines from cultured human dermal keratinocytes, fibroblasts and microvascular endothelial cells [20, 21]. Both *D. farinae* and *D. pteronyssinus* extracts stimulate cultured keratinocytes to increase secretion of the chemokine growth-related oncogene- α (GRO α , CXCL1), which is chemotactic for the extravasation of leukocytes during inflammation [20]. Likewise, *D. farinae* and/or *D. pteronyssinus* extracts induce cultured normal human dermal fibroblasts to secrete interleukin (IL)-6, IL-8 (CXCL8), monocyte chemoattractant protein-1 (MCP-1, CCL2) and macrophage colony-stimulating factor (M-CSF) [20]. Kato et al. [22] demonstrated that whole-mite culture extract and recombinant Der f 1 and Der p 1 induced the release of IL-8 and granulocyte/macrophage colony-stimulating factor (GM-CSF) from cultured primary human keratinocytes from infant foreskins and from the human keratinocyte cell line HaCaT. In these previous studies, the effect of mite extracts was

investigated using monocultures of epidermal keratinocytes or dermal fibroblasts. In vivo in the skin, fibroblasts and keratinocytes may respond differently to mite extracts because the cytokines secreted by one cell type can influence the function of the others and of the fibroblasts that are associated with a collagen matrix [12, 23]. The cytokines that these 2 cell types produce can mediate the function of many other cells in the skin, including microvascular endothelial cells, which are key to the regulation of extravasation of inflammatory and immune cells. Therefore, the responses of keratinocytes and fibroblasts are important in the progression of inflammatory and immune responses and the manifestation of atopic dermatitis. Here, we report the effect of house dust mite extracts on fibroblasts and keratinocytes together in human skin equivalents (HSEs). The HSE is structurally similar to normal skin. It consists of an epidermis with a stratum corneum over living keratinocytes and a basal layer grown over a dermis consisting of fibroblasts in a collagen matrix [23, 24].

Methods

Mite Extracts

The house dust mites *D. farinae*, *D. pteronyssinus* and *E. maynei* were cultured in our laboratory at 75% relative humidity and room temperature. Mites were collected as they migrated from thriving cultures and were frozen until use. Soluble protein extracts were prepared by extracting a weighed amount of mites in endotoxin-free water (Lonza, Walkersville, Md., USA) for 24 h at 4°C. Samples were sonicated for 15 min on ice then ground in a TenBroeck homogenizer, and insoluble material was removed by centrifugation. The supernatant (extract) was collected and sterile-filtered into sterile vials. Protein content was measured using the Bradford protein assay with bovine serum albumin as the standard [25]. Endotoxin content was determined using a Limulus Amebocyte Lysate QCL-1000 assay kit from Lonza according to the directions provided.

HSE Challenge

EpiDerm EFT-400 full-thickness HSEs and medium were purchased from MatTek Corp. (Ashland, Mass., USA). Upon arrival, HSEs on their supports were transferred to new 6-well culture plates containing 3.0 ml of fresh medium per well, and plates were placed in a 37°C incubator with 5–7% CO₂. The next day, HSEs were transferred to new plates containing 5.0 ml of fresh medium. The epidermal surfaces of HSEs were inoculated with 100 μ g of mite extract protein (26–38 μ l), while the surfaces of control HSEs were not inoculated. There were 3 replicate HSEs for each test condition. At 6, 12, 18 and 24 h after inoculation, each HSE on its support was lifted from the well, the medium was mixed and a 550- μ l aliquot was removed from the sample well and frozen at –80°C. At the conclusion of the experiment (after 48 h), the remaining medium was collected and frozen.

Table 1. Immunomodulation activities of house dust mite extracts on normal human skin keratinocytes and fibroblasts in monocultures (24 h) and HSEs (48 h)

	<i>D. farinae</i> extract			<i>D. pteronyssinus</i> extract			<i>E. maynei</i> extract		
	Cell type: keratinocytes Inoculum: 100 µg/ml (in media)	fibroblasts 100 µg/ml (in media)	HSEs 100 µg (on surface)	keratinocytes 100 µg/ml (in media)	fibroblasts 100 µg/ml (in media)	HSEs 100 µg (on surface)	keratinocytes 100 µg/ml (in media)	fibroblasts 100 µg/ml (in media)	HSEs 100 µg (on surface)
CTACK	X	X	>	X	X	>	X	X	=
TSLP	X	X	=	X	X	=	X	X	=
GROα	= ^a	X	=	= ^a	X	=	< ^a	X	=
TGFα	= ^a	X	>	= ^a	X	>	< ^a	X	>
IL-1α	X	0 ^a	=	X	0 ^a	>	X	0 ^a	>
IL-1β	0 ^a	0 ^a	X	0 ^a	0 ^a	X	0 ^a	0 ^a	X
IL-1ra	= ^a	0 ^a	=	= ^a	0 ^a	>	= ^a	0 ^a	=
IL-3	0 ^a	X	X	0 ^a	X	X	0 ^a	X	X
IL-6	0 ^a	> ^a	>	0 ^a	= ^a	>	0 ^a	= ^a	=
IL-7	0 ^a	0 ^a	X	0 ^a	0 ^a	X	0 ^a	0 ^a	X
IL-8	> ^b	> ^a	=	> ^b	> ^a	>	> ^b	> ^a	>
IL-10	0 ^a	X	X	0 ^a	X	X	0 ^a	X	X
IL-12p7	0 ^a	X	X	0 ^a	X	X	0 ^a	X	X
IL-15	0 ^a	0 ^a	X	0 ^a	0 ^a	X	0 ^a	0 ^a	X
MCP-1	0 ^a	> ^a	=	0 ^a	= ^a	=	0 ^a	= ^a	=
G-CSF	X	0 ^a	=	X	0 ^a	=	X	0 ^a	=
GM-CSF	0 ^a	0 ^a	=	0 ^a	0 ^a	>	0 ^a	0 ^a	>
M-CSF	X	> ^a	=	X	> ^a	>	X	= ^a	>
VEGF	X	0 ^b	>	X	0 ^b	=	X	0 ^b	=
Eotaxin	X	0 ^a	X	X	0 ^a	X	X	0 ^a	X
SCF	X	0 ^a	X	X	0 ^a	X	X	0 ^a	X
TNFα	X	0 ^a	X	X	0 ^a	X	X	0 ^a	X

Data are compared to paired constitutive controls and are scored as equivalent to (=), downmodulated (<) or upmodulated (>). Some cytokines were not detected (0) while others were not measured (X). Keratinocytes and fibroblasts were monocultures. Collection sites were media in wells for all extracts. HSE data are from this study at 48 h. SCF = Stem cell factor; TNFα = tumor necrosis factor-α.

^a Data from Arlian et al. [20].

^b Data from Arlian et al. [unpubl.].

Cytokine Measurements

The concentrations of various cytokines in the culture medium aliquots were measured using ELISA kits from R&D Systems (Minneapolis, Minn., USA). The cytokines selected for assay in this study were those that we had previously detected in supernatants of monocultures of keratinocytes or fibroblasts challenged with dust mite extracts (table 1) [20]. Data are presented as means ± SEM. Statistical significance was determined using a single-factor analysis of variance of differences compared to controls, with $p < 0.05$ judged as significant.

Results

Endotoxin and Protein Concentrations of Extracts

The endotoxin concentrations of the mite extracts were 4.57×10^6 , 1.15×10^6 and 10.2×10^6 endotoxin units (EU)/ml for *D. farinae*, *D. pteronyssinus* and *E. maynei*, respectively.

The endotoxin concentration of *D. farinae* extract was 4.0 times greater than that of *D. pteronyssinus*. The endotoxin concentration of *E. maynei* was 2.2 and 8.9 times greater than that of *D. farinae* and *D. pteronyssinus*, respectively.

Protein concentrations in the extracts of *D. farinae*, *D. pteronyssinus* and *E. maynei* were 3.07, 3.81 and 2.64 mg/ml, respectively. Therefore, HSEs inoculated with 100 µg of extract protein were also inoculated with 149,000 EU from *D. farinae*, 30,100 EU from *D. pteronyssinus* or 386,000 EU from *E. maynei*.

Control HSEs

Uninoculated HSEs constitutively secreted cutaneous T cell-attracting chemokine (CTACK, CCL27), GROα, IL-6, IL-8, MCP-1, granulocyte colony-stimulating factor

(G-CSF), GM-CSF, M-CSF and vascular endothelial cell growth factor (VEGF), and the levels of these cytokines in the media beneath these control samples increased during the 48-hour experimental period (fig. 1). Conversely, no thymic stromal lymphopoietin (TSLP), transforming growth factor- α (TGF α) or IL-1 α was detected in media from control HSEs at any time during the experiment. IL-1 receptor antagonist (IL-1ra) was present in the medium used to culture the HSEs, and its level dropped in medium collected from the uninoculated HSEs over the course of the experiment.

Inoculated HSEs

TGF α . *D. farinae*, *D. pteronyssinus* and *E. maynei* extracts induced the secretion of small but significant amounts of TGF α (fig. 1). This was the only cytokine significantly upregulated by extracts of all 3 mite species.

IL-6 and IL-8. HSEs constitutively secreted copious amounts of both IL-6 and IL-8. *D. pteronyssinus* extract significantly increased the secretion of these cytokines above control levels (fig. 1). *D. farinae* extract induced significantly increased secretion of IL-6 but not of IL-8. Conversely, *E. maynei* extract induced significantly increased secretion of IL-8 but not IL-6.

Colony-Stimulating Factors. Extracts of *D. pteronyssinus* and *E. maynei* upregulated production of GM-CSF and M-CSF (fig. 1). *D. farinae* extract did not induce significant secretion of either of these factors. No dust mite extract significantly upregulated G-CSF levels.

VEGF. Secretion of VEGF was enhanced by stimulation with extracts of *D. farinae* but not by extracts of *D. pteronyssinus* or *E. maynei* (fig. 1).

IL-1 α and IL-1ra. IL-1 α was not constitutively secreted by uninoculated HSEs, but extracts of *D. pteronyssinus* and *E. maynei* induced significant production of some IL-1 α (fig. 1). The medium used to culture the HSEs had considerable amounts of IL-1ra, but compared to control HSEs, only those challenged with *D. pteronyssinus* extract had significantly elevated levels of this substance at the end of the experiment.

CTACK. Extracts of the *Dermatophagoides* mites significantly upregulated CTACK production above control levels (fig. 1).

TSLP, GRO α and MCP-1. TSLP was not constitutively produced by HSEs, and only trace amounts of it were detected in response to stimulation with any mite extract. GRO α and MCP-1 levels increased over time in the medium beneath all HSEs, but none of the mite extracts induced secretion levels that were significantly different from those of control tissues (constitutive secretion; fig. 1).

Discussion

Secretion of cytokines and chemokines by epidermal keratinocytes and dermal fibroblasts can play a major role in the pathogenesis of the skin, including atopic dermatitis. A double-blind controlled trial has shown that effective house dust mite avoidance measures greatly reduce the activity of atopic dermatitis [26]. Previously, we found that molecules in house dust mite extracts could modulate the secretion of proinflammatory cytokines and chemokines secreted by cultured normal keratinocytes and fibroblasts [20] (table 1). Specifically, keratinocytes stimulated with *E. maynei* extract downregulated the constitutive secretion of TGF α and GRO α [20]. Cultured fibroblasts stimulated with *D. farinae* increased secretion of IL-6, IL-8, MCP-1 and M-CSF [20]. In contrast, cultured fibroblasts stimulated with *D. pteronyssinus* increased secretion of IL-8 and M-CSF but not of IL-6 or MCP-1, while *E. maynei* extract only stimulated increased IL-8 secretion. In this previous study, the cells stimulated with mite extract were monocultures of either keratinocytes or fibroblasts. Thus, those cells responded independently of other stimuli such as cytokines, chemokines and physical contact with other cell types that are present in vivo in their natural environment. Therefore, in the present study, we stimulated HSEs that contained both keratinocytes and fibroblasts in a collagen matrix to see if cell and matrix interactions as they occur in vivo would result in similar responses.

A significant finding in the current study was that the responses of keratinocytes and fibroblasts together in the HSE model in some cases were different from those of the isolated cultured cells. Cells in HSEs (fibroblasts and keratinocytes together) secreted IL-1ra, TGF α and GM-CSF in response to extract of at least 1 mite species, but fibroblasts and/or keratinocytes in monoculture did not (table 1). GM-CSF was undetectable in monocultures, but significant levels were secreted by HSEs. Keratinocyte monocultures did not secrete MCP-1 constitutively or when stimulated with any mite extract, while fibroblast monocultures secreted only small amounts (<200 pg/ml). However, when keratinocytes and fibroblasts were cocultured in the HSE format, substantial amounts of MCP-1 were secreted. HSEs secreted IL-6, IL-8 and M-CSF at levels similar to monocultured fibroblasts. Cultured keratinocytes, like HSEs, secreted IL-8. Clearly, the cell response was modified by the interaction of cells and matrix in the HSE. It is interesting that HSEs, keratinocytes and fibroblasts all secreted IL-8 in response to extracts of all 3 species of house dust mite. IL-8 is a chemo-

Fig. 1. Time course of cytokine secretion by HSEs in response to surface inoculation with 100 µg of house dust mite extract protein from *D. fariniae* (DF), *D. pteronyssinus* (DP) or *E. maynei* (EM).

kine that is chemotactic for neutrophils and promotes their extravasation from the bloodstream into the dermis.

Consistent with our previous findings using monocultures of cells, another important finding of this research was that the extracts made from different mite species could induce different responses. Both *D. farinae* and *D. pteronyssinus* extracts stimulated significant IL-6 and CTACK secretion by HSEs but *E. maynei* did not. *D. pteronyssinus* and *E. maynei* significantly stimulated IL-1 α , IL-8, M-CSF and GM-CSF secretion but *D. farinae* did not. *D. farinae* stimulated secretion of VEGF but neither *D. pteronyssinus* nor *E. maynei* did. It is not clear what mechanism underlies these differential responses. It is likely that the extracts from the 3 different mite species contain different molecules or different quantities of similar molecules. If different molecules are present in the extract, these different molecules may stimulate the cells via different receptors and mechanisms (pathways). Also, our results and those of previous studies show that the extracts of the different species contain different concentrations of endotoxins [27]. This may influence signaling via the toll-like receptor-4 pathway and others. However, the hierarchy of endotoxin concentrations was *E. maynei* > *D. farinae* > *D. pteronyssinus*, while the hierarchy of overall cytokine responses was *D. pteronyssinus* > *E. maynei* > *D. farinae*. This suggests that the responses were not induced primarily by endotoxin in the extracts. Likewise, mite extracts contain protease enzymes, and these may stimulate these cells through protease-activated receptors. Different quantities of protease enzymes and even differences in other enzymes may stimulate the cells differently via protease-activated receptors or other receptors.

A significant finding was that *D. farinae* and *D. pteronyssinus* extracts induced the secretion of significantly increased levels of CTACK, presumably from keratinocytes. CTACK from keratinocytes plays an important role in attracting subpopulations of memory lymphocytes to the skin [28]. It is interesting that *E. maynei* extract did not significantly upregulate secretion of CTACK. It is also noteworthy that all 3 mite extracts induced the secretion of the proinflammatory cytokine IL-1 α by HSEs, but the levels induced by *D. pteronyssinus* and *E. maynei* were significantly increased and were 4–8 times the levels elicited by *D. farinae* extract. We did not see any secretion of IL-1 α by monocultured fibroblasts. However, based on the HSE data, it appears that mite material of some species can induce the synthesis and release of IL-1 α , probably from keratinocytes, which can contribute to

skin inflammation. It is interesting that HSEs released significantly increased levels of IL-1ra in response to only *D. pteronyssinus* and not *D. farinae* or *E. maynei*. IL-1ra is a competitive inhibitor of IL-1 by blocking the IL-1 receptor and thus has anti-inflammatory activity. However, in response to the burrowing of the related scabies mite, *Sarcoptes scabiei*, on their surface, HSEs increased the release of IL-1ra [23]. This could be a response to salivary secretions, fecal material or physical activity from these parasitic mites. This may be an adaptation that the parasite uses to depress the host's inflammatory response and that allows the parasite to reproduce and become established in the host's skin.

An interesting finding of this study was that, in many cases, *D. pteronyssinus* and *E. maynei* extracts induced much greater cytokine secretion than did *D. farinae*. The significance of this is not clear and requires further investigation. However, the data suggest that *D. pteronyssinus* and *E. maynei* may be capable of causing more severe inflammatory reactions in the skin than *D. farinae*.

Our studies with the HSE model have allowed us to investigate cell and matrix interactions that more closely resemble tissue in vivo. The different responses by pure cultured cells and skin equivalent tissue illustrate the danger of using cultured cells to determine the overall effect of dust mites on cells that are involved in allergic and asthmatic reactions. Many studies have stimulated lung epithelial cells in culture with house dust mite extract or recombinant house dust mite allergen [19, 29–35]. These cells produce many cytokines in response to dust mite molecules. These studies have shown that molecules in house dust mite extracts or specific allergens stimulate lung epithelial cells to secrete cytokines, including IL-6, IL-8, GM-CSF and eotaxin (CCL11) [29–37]. These same cytokines and chemokines are released by cultured fibroblasts and keratinocytes and by the cells in HSEs. It is not known if these lung/bronchial cells give similar responses in vivo when they interact via cytokines and contact with other cells and a matrix. Given that we have demonstrated that isolated cells behave differently and give different responses than cells in a tissue, this must be considered in the interpretation of these data and the role of these cytokines in airway inflammation and asthma.

It is not clear if the house dust mite extracts used in the present study induced the release of stored cytokines and chemokines from keratinocytes and fibroblasts or if they actually induced the expression of mRNA for the synthesis of these proteins and then their release. Additional study is needed to determine this.

Conclusion

Our results using HSEs along with our previous results using monocultured cells show that molecules from house dust mites are capable of inducing the release of multiple proinflammatory cytokines and chemokines from epidermal keratinocytes and dermal fibroblasts. Secretion of cytokines and chemokines by keratinocytes and fibroblasts can play a major role in the pathogenesis of the skin, including atopic dermatitis. The responses we observed in these skin cells were innate responses, because the HSEs contained no immune effector cells such as lymphocytes, mast cells or Langerhans cells. However, chemokines and cytokines from keratinocytes and fibroblasts play a role in the events that lead to the activation and infiltration of inflammatory and immune cells into

the skin. House dust mites can induce skin keratinocytes and fibroblasts to secrete cytokines that promote inflammation, and this may contribute to atopic dermatitis and other skin diseases. Avoiding skin contact with house dust mite material would eliminate this possibility. Frequent vacuuming of couches, furniture and carpets and frequent laundering of sheets and other bedding and clothing to remove mite molecules, including allergens, would help reduce the possibility of skin contact.

Acknowledgement

This work was supported by a grant to L.G.A. from the National Institutes of Health, National Institute of Allergy and Infectious Diseases (NIAID grant AI-017252).

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