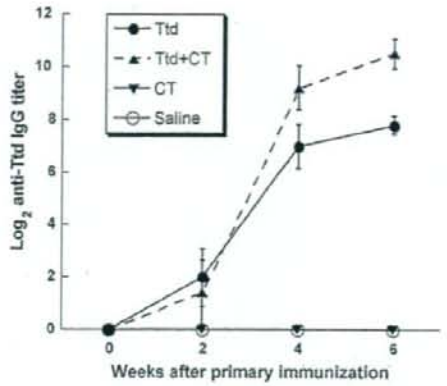

Fig. 8. Immunization using the tetanus toxoid (Ttd) for the prolonged TCI procedure with or without adjuvant. The ears of C57BL/6 mice ($n=5$, but $n=4$ for the "with adjuvant" group) were immunized by the prolonged TCI procedure for 16 h with 10 Lf of Ttd with (closed triangles and a broken line) or without (closed circles and an unbroken line) 10 μg of CT as an adjuvant at 0, 2, and 4 weeks. The negative controls were immunized with 10 μg of CT (reverse closed triangles and an unbroken line) or vehicle (PBS) alone (open circles and an unbroken line) in an identical manner. OVA-specific IgG titres in serum samples were determined every 2 weeks for up to 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group and time.

3.6. Prolonged TCI using the Ttd induced a robust antibody response and provided protection against tetanus toxin challenge

We immunized the ears of the C57BL/6 mice with 10 flocculation units (Lf) of Ttd with or without 10 μg of CT according to the prolonged TCI procedure. As controls, we also immunized another group of mice with 10 μg of CT or vehicle (PBS) alone. We boosted the mice twice at 2-week intervals in a manner identical to that used for the primary immunization. We collected serum samples every alternate week for up to 6 weeks after the primary immunization and determined the titres of Ttd-specific IgG antibodies in the sera. Regardless of the use of the adjuvant, significant levels of Ttd-specific IgG antibodies were induced in the sera of the mice immunized with the Ttd, and these levels increased after the booster immunizations (Fig. 8). In contrast, in the mice immunized with either the CT or vehicle alone, no Ttd-specific IgG antibodies were induced at all the times examined.

Next, we challenged these mice with a lethal dose of tetanus toxin at 7 weeks after the primary immunization. All the control mice (only CT or vehicle immunized) died within 2 days of the challenge with signs of tetanus (Table 1). In contrast, all the mice immunized with the Ttd survived and demonstrated no clinical signs regardless of adjuvant use.

Table 1
Tetanus toxin challenge[a]

	Survival/total	% Survival
Saline	0/5	0
CT	0/5	0
Ttd	5/5	100
Ttd + CT	4/4	100

[a] C57BL/6 mice were immunized by prolonged TCI procedure with 10 Lf of Ttd with or without 10 μg of CT at 0, 2, 4 weeks. Ten micrograms of CT or vehicle (saline) alone were immunized to the other groups of mice by the same way. Ten LD50 of tetanus toxins were challenged at 7 weeks after primary immunization.

4. Discussion

In the recent decade, many studies have demonstrated the feasibility of TCI using various antigens [3–7], adjuvants [10,13–16], skin treatments [7,17–19], and animals [20–24]. Under these various settings, most experiments followed a protocol in which antigens were topically applied for 1–2 h and reported the critical role of adjuvants for the induction of robust immune responses. Skin pretreatments such as skin abrasion [25], application of penetration enhancers [7,26], use of electrophoresis [17] or sonophoresis [18] techniques, and the use of lipid carriers [20,27] were applied in some experiments; these pretreatments might promote antigen penetration through the skin. Overall, some potent adjuvants and/or some skin penetration-enhancing methods are believed to be necessary to induce robust immune responses by TCI. Nevertheless, a few papers [28–30] reported that substantial immune responses were successfully induced in the absence of any adjuvants or penetration-enhancing methods. Further, in all of these experiments, the antigens were applied topically for a comparatively long- period of above over night. These results suggest that the duration of antigen presence on the skin during the TCI procedure might be an important parameter affecting the magnitude of the immune responses. However, thus far, the relationship between the duration of antigen presence on the skin and the magnitude of the immune response remains to be clearly elucidated. In this report, we applied OVA as an antigen for varying durations of 2–32 h on the intact skin of mice and observed that if the antigen was present on the skin for a prolonged duration, the serum antibody response was enhanced in a duration-dependent manner. Surprisingly, patch immunization on intact skin for above 16 h (referred to as prolonged TCI in this paper) induced substantial serum antibody responses even in the absence of any adjuvants. Dose–response and time-course experiments revealed that non-adjuvanted prolonged TCI to the mice ear induced serum antibody responses comparable in magnitude to those induced by adjuvanted prolonged TCI. Thus, our observations clearly indicated that the duration of antigen presence on the skin is an important factor influencing the effectiveness of TCI.

Several reports assume that some danger signals from bacterial adjuvants are necessary to activate Langerhans cells and trigger immune responses [8]. However, our data indicate that additional adjuvants are not prerequisites for the induction of immune responses. This result might imply that the antigen

itself stimulates the Langerhans cells or that some substances from skin bacterial flora play the role of danger signals. It is possible that a wet gauze patch stuck on the stratum corneum for a prolonged period stimulates the Langerhans cells. In fact, there are reports that some physical stimuli to the stratum corneum, such as tape-stripping or low frequency ultrasound, activate the Langerhans cells [18,37].

Our experiments clearly indicated that the ear skin was immunized more efficiently than the abdominal skin by the prolonged TCI procedure. It is known that the differences in skin structure, such as thickness of the stratum corneum and the density of Langerhans cells, among anatomic skin sites influence the penetration efficiency of low molecular percutaneous drugs [31,32]. The difference between the structures of the ear skin and abdominal skin may explain our observation. Thus, our observation indicates that skin anatomy is an important factor influencing the efficiency of TCI.

CT or LT has strong mucosal adjuvanticity. CT-adjuvanted or LT-adjuvanted TCI induces the mucosal antibody response, in addition to the systemic immune response [6,7,15,28,33,34]. Interestingly, we observed that non-adjuvanted prolonged TCI with OVA induced substantial antigen-specific IgG and IgA antibodies in the feces of mice although the magnitude of antibody production was significantly less as compared to that induced by CT-adjuvanted TCI. Our observation indicated that the mucosal immune response to TCI could be also induced even in the absence of CT or LT.

We observed substantial antibody responses in all the 3 strains of mice immunized by the prolonged non-adjuvanted TCI. This observation suggests that this modified TCI would be feasible for a wide range of genetic backgrounds. However, there were some significant differences among the 3 strains with regard to the magnitude of serum antibody productions. The C57BL/6 mice were the most sensitive to the prolonged TCI, while the C3H/He mice were the least sensitive. The response of the BALB/c mice was intermediate. Another group has previously reported a similar hierarchy in the sensitivity to TCI [38]. They used CT as an adjuvant and hypothesized that the hierarchy reflected the difference in the sensitivity to CT based on former experiments which indicated that the plasma IgG response to CT following oral or parenteral administration was under the genetic control of the I-A region of the H-2 major histocompatibility complex [39–41]. Here, we observed the same hierarchy regardless of the use of CT, suggesting that other mechanism(s) govern the sensitivity to TCI.

We applied the prolonged TCI method by using the Ttd as a model vaccine antigen and successfully induced significant serum antibody responses. Indeed, CT had an adjuvant effect in the prolonged TCI using Ttd, but non-adjuvanted prolonged TCI using the Ttd also induced substantial Ttd-specific antibodies in the sera of mice after the second booster immunization. The mice immunized with the Ttd according to the prolonged TCI method, adjuvanted and non-adjuvanted, survived with no clinical symptoms after challenge with a lethal dose of the tetanus toxin. These results suggest that non-adjuvanted prolonged TCI method is a feasible vaccination method for infectious diseases.

Thus far, the TCI method has been shown to be functional in various disease models, including bacterial and viral infections, malignancies [26,35], and Alzheimer's disease [36], using various animals, e.g., mice, rats [20], sheep [22], cattle [24], chickens [21], and humans [12,19]. To develop an effective TCI strategy for different aims, the TCI protocol with regard to antigens, adjuvants, skin treatments, etc. requires optimization. In this study, we pointed out the importance of the duration of antigen presence on the skin during the TCI procedure and showed that substantial antibodies were induced by non-adjuvanted TCI of 16-h duration. Our observations improve the understanding regarding the TCI mechanisms and offer a practical option for developing a safe and effective method for transcutaneous vaccination.

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Short communication

Detection of natural killer T cells in the sinus mucosa from asthmatics with chronic sinusitis

Background: Chronic sinusitis (CS) with asthma generally exhibits a high degree of sinus tissue eosinophilia and recurrence often occurs even after surgical therapy. However, the cause has not yet been fully clarified.
Aims of the study: To elucidate the pathogenesis of this refractory disease, we examined the infiltration of natural killer T (NKT) and type 1 helper T (Th1)/type 2 helper T (Th2) cells, and the cytokine expression in the sinus mucosa.
Methods: Sinus mucosal specimens were obtained surgically from 16 CS patients with nasal polyps. The NKT cells, Th1/Th2 cells and the expression of IL-4, IL-5, IL-13 and IFN-γ were examined by a polymerase chain reaction or flow cytometry. Nasal mucosal specimens from six other patients with allergic rhinitis (AR) were examined in a similar manner.
Results: The NKT cells were detected to varying degrees in the sinus mucosa from asthmatic CS patients, but neither in the nonasthmatics nor in the nasal mucosa from the patients with AR. The Th2 cells and Th2 cytokines were expressed at significantly higher levels in the sinus mucosa from the CS patients with asthma in comparison to those without asthma. However, the Th1 cell infiltration and IFN-γ expression were not different between these groups.
Conclusion: Natural killer T cells may, therefore, play important roles in the enhanced Th2 cytokine expression and increased infiltration of Th2 cells and eosinophils observed in the sinus mucosa from asthmatic CS patients through MHC-independent mechanisms.

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Key words: allergic rhinitis; asthma; chronic sinusitis; eosinophil; natural killer T cells

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Chronic sinusitis (CS) is a common disease that affects about 20% of the population, and is usually treated by conservative medical management (1). Many cases of CS with polyps in Japan predominantly exhibit an infiltration of neutrophils in the sinus and are generally responsive to macrolide therapy (2, 3). Although some cases of CS with sinus tissue neutrophilia (CSN) require surgical intervention, their prognosis is generally satisfactory (1, 4).

However, there is a severe subform of CS, which exhibits marked sinus infiltration with eosinophils and, to a lesser degree, neutrophils and other infiltrating cells. These cases of CS with sinus tissue eosinophilia (CSE) also exhibit the characteristics of multiple nasal polyps, relapse even after surgery, and a high degree of complication with asthma (5, 6). Although the involvement of fungal infection (7) and some bacterial toxins as allergens or superantigens has been reported (8), the exact mechanisms of development of CSE have not yet been ascertained. The involvement of a type 1 allergic reaction in CSE remains uncertain because it occurs infrequently with allergic rhinitis (AR) and CSE is more frequently linked to nonatopic rather than atopic asthma (9–11).

Human natural killer T (NKT) cells express the invariant Vα24JαQ paired with the Vβ11 antigen receptor and play important roles in innate immunity (12). Natural killer T cells are activated by a specific glycolipid antigen, α-galactosylceramide (α-GalCer), in a CD1d-dependent manner (13). CD1d is an HLA class I-like antigen priming molecule that does not exhibit any allelic polymorphism. Therefore, the NKT activity is not restricted to MHC antigens. Recently, the involvement of NKT cells in the development of airway hypersensitivity in mice (14) and the detection of NKT cells in bronchoalveolar-lavage fluid samples from patients with moderate to severe asthma (15) were reported.

In the present study, we examined NKT cells, as well as Th1/Th2 cells and the expression of various cytokines in

Abbreviations: α-GalCer, α-galactosylceramide; AR, allergic rhinitis; CS, chronic sinusitis; CSE, chronic sinusitis with sinus tissue eosinophilia; CSN, chronic sinusitis with sinus tissue neutrophilia; NKT cell, natural killer T cell.

the sinus mucosa from asthmatic and nonasthmatic CS patients.

Methods

Patients and samples

All patients included in this study complained of CS with nasal polyps, were operated at Chiba University Hospital from January to October 2006. Chronic sinusitis was clinically determined as the presence of a combination of major symptoms (such as nasal discharge, nasal obstruction, hyposimia, facial congestion, facial fullness) and computed tomography (CT) findings. The symptoms were scored in all patients. The symptom scoring range in this study had four options as reported previously (16, 17). The exclusion criteria included cystic fibrosis, immotile cilia syndrome, autoimmune diseases and being positive response to HIV, hepatitis C virus and for hepatitis B surface antigens. The diagnosis of asthma was based on the American Thoracic Society criteria (18) and patient with aspirin-intolerant asthma was proven to have histories of severe bronchoconstriction after the ingestion of at least two different nonsteroidal anti-inflammatory drugs.

All patients received various medications including anti-histamines, anti-leukotrienes, and local or systemic steroids but failed to show any significant improvements. All medications were withdrawn for 1 week prior to the operation, however, the one patient (#15 in Table 1) could not stop using oral steroids because of asthma. A preoperative examination included allergen specific IgE (mite, cedar, house dust, Candida, Alternaria by CAP radio-allergo sorbent test) and blood eosinophil counts by using a standard automated cell counter. Surgical biopsy samples from the ethmoidal sinus mucosa, including polyps, were obtained and examined for inflammatory cells by hematoxylin and eosin staining. The sinus mucosa specimens from each patient were immediately cut into small pieces and incubated with PBS containing 5% fetal calf serum (FCS), 1 mg/ml collagenase A (Roche, Mannheim, Germany) and 0.2 mg/ml DNase I (Sigma-Aldrich, St Louis, Mo, USA) at 37°C for 35 min. After incubation with 20 mM EDTA for 5 min at room temperature, the specimens were passed through a fine nylon mesh. Mononuclear cells were subsequently obtained by the Ficoll–Hypaque technique and were used for the polymerase chain reaction (PCR) assay. For the flow cytometric analysis, collagenase and DNase I were not used during the preparation of the mononuclear cells to avoid interference with the cell surface markers.

Another six patients with serious perennial AR due to *Dermatophagoides pteronyssinus* were also enrolled in this study. The diagnosis of AR was based on the criteria of Okuda et al. (19), namely, typical perennial allergic nasal symptoms and a positive CAP radio-allergo sorbent test

Table 1. Profile of chronic sinusitis (CS) and allergic rhinitis (AR) patients

	Patient	Age	Sex	Blood eosinophils (%)	AR	CS with polyps	Tissue eosinophils*	Tissue neutrophils*	E/N ratio†	CS Score‡
CS without asthma	1	54	M	n.t.	–	+	5	90	0.06	2.6
	2	56	M	1.1	–	+	4	120	0.03	2.4
	3	55	M	2.6	–	+	6	180	0.03	2.6
	4	77	M	1.0	–	+	2	110	0.02	2.6
	5	38	M	3.7	–	+	12	400	0.03	2.8
	6	65	M	2.5	–	+	3	140	0.02	2.6
	7	71	F	1.8	–	+	2	70	0.03	2.8
	8	64	M	n.t.	–	+	3	120	0.03	2.4
	9	32	M	3.4	+	+	6	150	0.04	2.6
	10	57	M	6.6	+	+	135	50	2.7	2.8
CS with asthma	11	43	F	1.8	–	+	42	120	0.35	2.8
	12	61	M	11.2	–	+	>500	110	>4.5	3
	13	25	M	4.7	–	+	125	50	2.5	2.6
	14	57	F	16.9	–	+	190	55	3.5	2.8
	15	45	M	2.6	–	+	330	80	4.1	2.6
	16	56	F	8.0	–	+	>500	100	>5.0	2.8
AR	17	53	M	4.9	+	–				
	18	66	M	7.1	+	–				
	19	25	F	14.7	+	–				
	20	37	M	7.1	+	–				
	21	54	F	n.t.	+	–				
	22	22	M	4.9	+	–				

n.t., not tested.

*Number of eosinophils and neutrophils/×400 magnification.

†Number of tissue eosinophils/number of tissue neutrophils.

‡Severity scoring of CS major symptoms (from ref. 17).

result against *D. pteronyssinus*. The inferior nasal turbinate mucosa was obtained surgically and was examined in the same manner.

The study received prior approval from the Ethics Committee of the Chiba University. Informed consent was obtained from each patient.

PCR analysis

Total RNA was isolated from the mononuclear cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using oligo primers and Superscript II RT (Invitrogen). The specific primers for the detection of IL-4, IL-5, IL-13, IFN-γ CD3, GAPDH were purchased from Applied Biosystems (Foster City, CA, USA). For the detection of Vα24Jα18, the forward primer 5′-CCTCCCAGCTCAGCGATTC-3′, the reverse primer 5′-TATAGCCTCCCCAGGGTTGA-3′ and the probe FAM-5′-CCTCCTACATCTGTGTGGTGAGCGACA-3′-TAMtph were used (20). The samples were subjected to a real-time PCR analysis on an ABI PRISM7000 Sequence Detection system.

Flow cytometric analysis

The mononuclear cells were incubated on ice with the appropriate staining reagents: fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (mAb), R-phycoerythrin (R-PE)-conjugated anti-CCR4 mAb, allophycocyanin (APC)-conjugated anti-CXCR3 mAb, or FITC-, RPE- or APC-conjugated isotype match control mAb (BD Bioscience, San Jose, CA, USA) (21).

The cells were subjected to a flow cytometric analysis using a flow cytometer (FACS Calibur: BD, Franklin Lakes, NJ, USA) and the results were analyzed using the FlowJo software program (Tree Star, Inc, Ashland, OR, USA).

Statistical analysis

A statistical analysis was performed using the Wilcoxon rank sum test or the Wilcoxon signed rank test for paired and unpaired data. *P*-values < 0.05 were considered to be statistically significant.

Results

A total of 16 patients were analyzed. All patients had a severe loss of smell and nasal obstruction. Allergic fungal sinusitis with eosinophil granule-rich mucin and diagnostic clinical characteristics (17) was not detected in these patients. Six patients had asthma and two of them were aspirin-intolerant asthma (#12, 16 in Table 1). All these asthma patients were negative for specific IgE to inhalant allergens.

Based on the number of sinus mucosal eosinophils, the CS patients were divided into CSE [number of eosinophils/number of neutrophils (E/N ratio) > 1.0] and CSN (E/N ratio < 1.0). Of the 16 patients with CS, six were considered to be CSE and ten were CSN. Five of the six CSE patients had asthma and one had AR but not asthma. Of the 10 patients with CSN, one had asthma and one had AR.

IL-5 mRNA expression was observed in the mononuclear cells isolated from the sinus mucosa from all patients with asthma or with CSE and the expression was significantly higher than in patients without either asthma or with CSN. These findings were similar to the expression of IL-4 and IL-13 (Fig. 1). Although IFN-γ was detected in the sinus mucosa from each of the patients, there was no significant difference between patients with and without asthma (data not shown).

In the flow cytometric analysis of the sinus mucosa, the number of CXCR3-positive T cells was not different

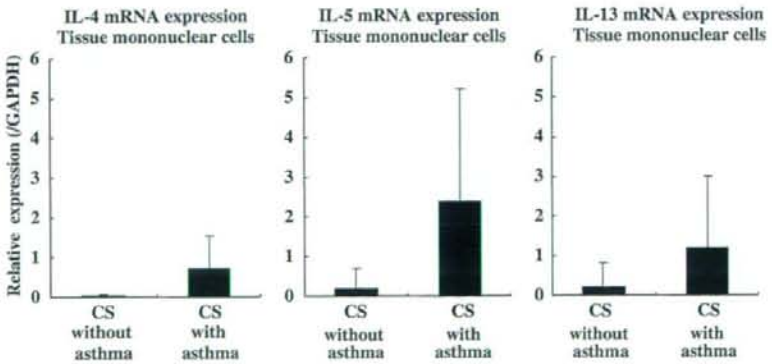

Figure 1. IL-4, IL-5 and IL-13 mRNA expression in mononuclear cells isolated from sinus mucosa in chronic sinusitis patients. Significantly increased expression was observed in patients with asthma by real time PCR. This indicated that the T cells produced significant levels of IL-4, IL-5 and IL-13.

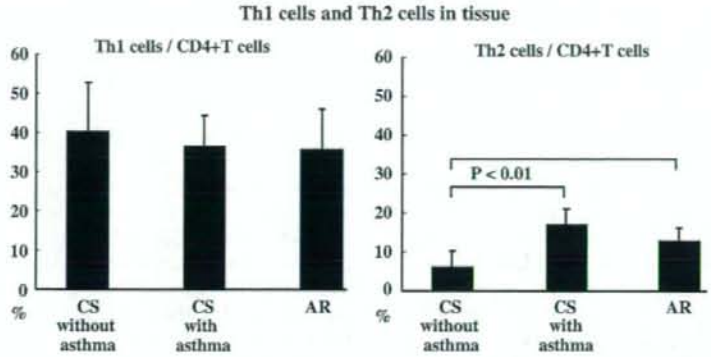

Figure 2. The ratio of CXCR3-positive and CCR4-positive T cells to CD4-positive T cells in mucosal specimens from chronic sinusitis (CS) patients with or without asthma, or in nasal mucosa from patients with allergic rhinitis (AR). Although the ratio of CXCR3-positive T cells were not different among these groups of patients, CCR4-positive T cells were increased significantly in asthmatic CS and AR patients compared with the nonasthmatic CS patients.

between the patients with and without asthma, nor between CSE and CSN. However, a significantly greater number of CCR4-positive T cells detected in patients with asthma or with CSE in comparison to those without either asthma or with CSN (Fig. 2).

NKT cells were detected at various levels in the peripheral blood of all CS patients, however, in sinus mucosa NKT cells were only detected in the patients with asthma. Five out of six patients with CSE and only one patient with CSN exhibited NKT cells in the sinus mucosa (Fig. 3). The patient with CSE who did not exhibit NKT cells in the sinus mucosa had perennial AR due to mites, but not asthma. The patient with CSN exhibited NKT cells in the sinus mucosa, combined with asthma, and an expression of Th2 cytokines and infiltration of CCR4-positive T cells was observed in the sinus mucosa. The expression levels did not correlate with the severity of CS.

In the inferior nasal turbinate mucosa from the six patients with AR, the CCR4-positive T cells were as high as those in the CSE patients, and the CXCR3-positive T cells were similar to those of CSN and CSE. However, NKT cells were not detected in the nasal mucosa from any of the patients with AR (Fig. 3).

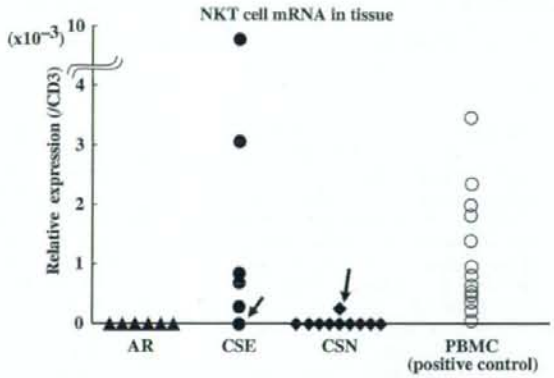

Figure 3. The relative expression of natural killer T (NKT) cells to CD3 T cells. NKT cells were detected in the sinus mucosa from five of six chronic sinusitis with sinus tissue eosinophilia (CSE) patients and one of 10 chronic sinusitis with sinus tissue neutrophilia (CSN) patients. The one CSE patient (indicated by the arrow) who was negative for NKT cells did not have asthma, but exhibited allergic rhinitis. The single CSN patient (indicated by the arrow) who was positive for NKT cells had asthma.

Discussion

In the present study, a significantly more number of Th2 cells, and consequently, expression of Th2 cytokines, was observed in the sinus mucosa from patients with asthma or with CSE. The low level of expression of Th2 cytokines and infiltration of Th2 cells in the sinus mucosa among the patients without asthma or with CSN, along with the fact that there was no difference in the Th1 cell infiltration among these sinus mucosa, altogether indicate that the Th2-predominant state in the sinus mucosa is characteristic of CS with asthma and of CSE.

A similar Th2-predominant state was observed in the nasal mucosa from patients with AR, which is a typical type I allergic disease mediated through allergen-specific IgE antibodies, although the pathogenesis of CSE is considerably different. The complication rate of AR in CSE is not high, and highly complicated asthma with CSE is nonatopic rather than atopic (9–11).

In this study, we detected NKT cells at various levels in the PBMCs from all patients, with no significant difference between the CSE and CSN patients. In the sinus mucosa, NKT cells were recognized in all the patients with asthma, in five out of the six patients with CSE and

in one CSN patient. The patient with CSN who was positive for NKT cells in the sinus mucosa had asthma and exhibited some degree of infiltration of eosinophils and Th2 cells, despite the predominant infiltration of neutrophils. In contrast, the patient with CSE who was negative for NKT cells in the sinus mucosa had AR and a Th2-predominant state in the sinus mucosa, but this was not combined with asthma. Natural killer T cells were not detected in the sinus mucosa from the CS patients without asthma, or in the nasal mucosa from the AR patients. These results suggest that NKT cells are not directly related to the development of allergy, but they may play important roles in the development of sinus disease combined with asthma as 'one airway, one disease (22)', and substantially different mechanisms must exist between allergy and the accumulation of eosinophils in the sinus mucosa in CSE. In a recent study with mice, Meyer et al. (23) demonstrated eosinophil accumulation in the airway and the development of airway hyperreactivity following the respiratory administration of α-GalCer, a specific NKT cell-activating glycolipid antigen, and that eosinophil accumulation was observed in the respiratory tract even in MHC class II-deficient mice. In the present study, we clearly demonstrated the presence of NKT cells in the sinus mucosa from CS patients with asthma, but not from CSE patients without asthma, nor in the nasal mucosa from AR patients. Chronic sinusitis with sinus tissue eosinophilia may be composed of several diseases with different etiologies. Natural killer T cells were detected in the sinus mucosa from CS patients with asthma and are, therefore, suggested to participate in the local immune reactions of the sinus mucosa. The elucidation of the mechanisms of NKT cell activation in the sinus mucosa and its relationship to the process of tissue eosinophilia may, therefore, lead to the fundamental resolution of these refractory diseases, including asthma.

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ORIGINAL ARTICLE

Preliminary Study on Japanese Cedar Pollinosis in an Artificial Exposure Chamber (OHIO Chamber)

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ABSTRACT

Background: A pollen exposure chamber (OHIO Chamber) was built in central Tokyo, Japan, in order to study seasonal allergic rhinitis (SAR). Since satisfactory outcomes were obtained from the controlled pollen exposure at the chamber, we conducted preliminary studies in volunteers with SAR.

Methods: Ten volunteers with SAR sensitive to Japanese cedar (JC) pollen were enrolled in this study. In order to investigate the intranasal and intraocular pollen number, volunteers were initially exposed to a low concentration of JC (2500 grains/m^3) for at most 1 hour in this chamber. Before and after the exposure, nasal cavities and eyes were washed with 100 ml and 25 ml of saline, respectively. Nasal and eye washing solutions were collected and the number of JC pollen was counted.

After 3 hours the volunteers were subsequently exposed to a moderate concentration of JC (4500 grains/m3) for 2 hours. Subjective nasal and ocular symptoms were recorded and the amount of nasal secretion was measured during the allergen exposure periods.

Results: During the initial exposure, all volunteers except one stayed in the chamber for 1 hour without any nasal or ocular symptoms. The number of pollen in the nose and eyes was 249.2 ± 120.9 and 13.6 ± 13.6 grains, respectively.

During the subsequent 2-hour exposure to JC pollen, nasal and ocular symptoms developed gradually in a time dependent manner in all the volunteers except one.

Conclusions: This is the first clinical study using Japanese cedar pollen under well-controlled conditions in the OHIO chamber in which the induction of allergic symptoms was observed. The OHIO chamber will be useful for studying allergic rhinitis in Japan.

KEY WORDS

allergen exposure, allergy symptoms, artificial exposure chamber, intranasal and intraocular pollen count, Japanese cedar pollen

INTRODUCTION

Japanese cedar (JC) pollinosis is a seasonal allergy, which is unique to Japan, and the causative allergen (JC pollen) is dispersed usually between February and April although the pollen count varies every year. Over 16% of the Japanese population suffer from the allergy during this season[1] and an incredible amount of anti-allergy agent is used, resulting in an undermined QOL[2] and decline in labor productivity.[3] This fact indicates that JC pollinosis is a social and economical problem which cannot be ignored.

Allergen exposure tests in natural environments have been conducted as fundamental research on this kind of allergy and also to examine the effectiveness of anti-allergy agents.[4,5] Studies of this sort, however, are greatly influenced by natural factors such as the amount of pollen, the weather, the temperature, or

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the wind velocity. The biggest problem is that the tests can be done only during the pollen season.

Several allergen exposure units have been built in Europe and the US to evaluate the efficacy of drugs and they are operating safely and effectively.[6-8] A predefined amount of pollen can be dispersed under stable conditions in these chambers.

Despite the fact that so many Japanese are allergic to JC pollen, this kind of chamber was not available in Japan before 2004. To meet the increased needs for such facilities, the first pollen exposure units in Japan were developed in Wakayama[9] in the first half of 2005. We built the third allergen exposure chamber, an artificial exposure chamber (AEC; OHIO Chamber), in the center of Tokyo in September, 2005. This chamber not only keeps the temperature and humidity constant, but also automatically cleans and dries the inside of the chamber.

Due to the lack of AECs in Japan, little fundamental data concerning Japanese cedar pollinosis were available up to this point. Since satisfactory outcomes were obtained from the controlled exposure at the OHIO Chamber, we were able to conduct preliminary studies on mildly symptomatic patients, in which we examined the amount of intranasal and intraocular pollen grains and the development of symptoms.

METHODS

SUBJECTS

The subjects of our study were mildly symptomatic adult patients. The inclusion criteria were as follows:

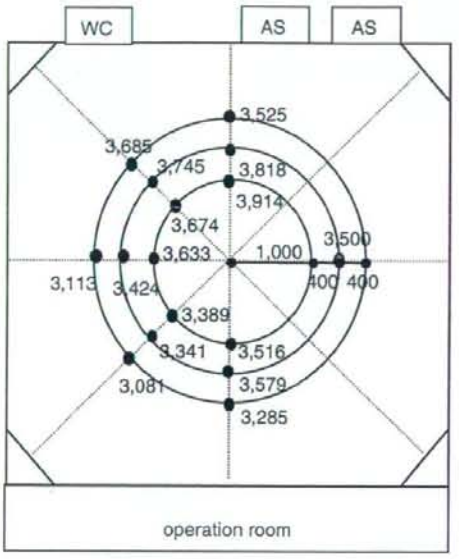
- Subjects must have at least a 2-year history of allergic symptoms during the pollen season, such as sneezing, nasal discharge, nasal obstruction, and itchy eyes.
- Subjects must also have had blood tests within 1.5 years showing positive RAST scores (≥Class 2) for JC pollen and negative RAST scores (≤Class 1) for house dust mite.

The exclusion criteria were as follows:

- Subjects with nasal obstruction attributable to a polyp or deformity of the nose.
- Subjects with acute upper respiratory infection(s) such as acute sinusitis, acute pharyngitis, and acute upper respiratory inflammation.
- Subjects with uncontrolled asthma, diabetes, high blood pressure, or eye diseases (glaucoma/cataract).
- Subjects who used anti-allergy agent(s) within a week before the start of this study.
- Pregnant women or women who trying to become pregnant.

The study was conducted in accordance with GCP Guidelines and the Declaration of Helsinki. The study was conducted on December 23 (2 months ahead of the start of the pollen season) after having been reviewed and approved by the ethics committee of Shinanozaka Clinic. Informed consent was obtained from all the subjects prior to study entry.

Fig. 1 Appearance of the artificial exposure chamber (OHIO Chamber). Pollen-diffuser in the center (arrow). Blower module in the corner of the room (arrow head)



Target concentration: 3500 grains/m³

WC: toilet AS: air shower

Fig. 2 Spatial concentration distributions of pollen in the OHIO Chamber at a height of 1.15 m at the target concentration of 3500 grains/m³. The average concentration of pollen was 3500 ± 419 grains/m³.

OHIO CHAMBER

The OHIO Chamber was installed in Samoncho Clinic in Yotsuya, the center of Tokyo. Its square measure is 25 m², the height 2.5 m, and the capacity 10 subjects (Fig. 1).

Compressed air transfers pollen grains from an outside dust feeder into the operation room where it is mixed with conditioned air inside the pollen diffuser after which the mixed air jets out upward from the diffuser. The diffuser and the blower modules in

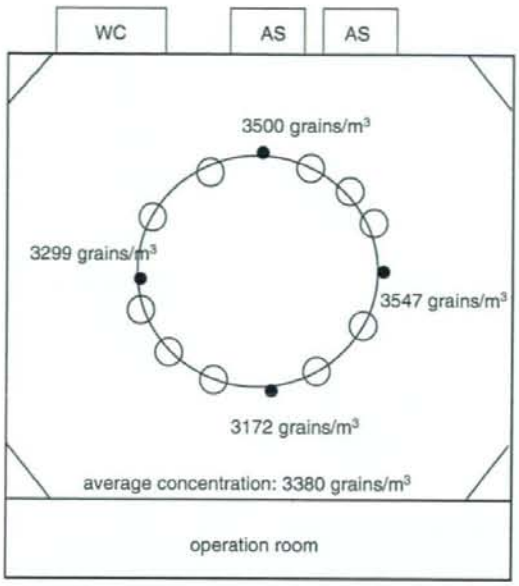


Fig. 3 The pollen concentration at a height of 1.15 m at the four points (●) when the ten volunteers (○) were present in the OHIO chamber. The average concentration was 3380 grains/m³ and uniform distribution of pollen was achieved.

four corners help the pollen grains get distributed evenly. The concentration of pollen grains in the room is measured by a particle counter equipped with a semiconductor laser (KC-20; Rion Co, Tokyo, Japan). This counter can absorb up to 30 L of air per minute and detects particles sized 10–100 μm.

Spatial distributions of pollen in the unmanned and manned chamber at a height of 1.15 m were measured. The pollen concentration distribution was within a range of ± 12% of the target value of 3500 grains/m³ in the unmanned chamber (Fig. 2). The pollen number at each of four points and the average pollen concentration are shown in Figure 3 when 10 subjects were present in the chamber.

The pollen count in this room is stable between 2500 and 120000 grains/m³ (data not shown). Also, the temperature inside the chamber was set at 22 ± 0.2℃ and the humidity was set about 20–55%. The pollen count, temperature, and humidity were monitored every 3 minutes and recorded accordingly.

Before entering the room, subjects were instructed to wear protection gowns so that the pollen grains were not attached to their hair or clothes. When entering or exiting, they also went through an air shower so as to avoid pollen grain contamination outside of the room. During the test, subjects were assigned to sit on chairs in designated areas of the chamber and in order to ensure the subjects' safety, their behavior was observed closely by staff and physicians situated in the operating room.

Table 1 Backgrounds of the subjects.

Subject No.	Sex	Age	RAST score
1	female	37	3
2	female	35	5
3	male	34	4
4	male	45	3
5	male	36	2
6	female	34	4
7	male	41	2
8	female	32	2
9	female	43	3
10	male	48	2

Five men and five women with mild symptoms were enrolled in this study.

This table shows their age and antibody titers (RAST scores) against cedar pollen.

STUDY DESIGN

This study consisted of two parts. In the first part, subjects were exposed to low-concentrated JC pollen (2500 grains/m³) in the chamber for up to an hour. They were allowed to exit the room if allergic symptoms developed (either in the nose or in the eyes). Immediately after exiting the room, all the subjects went through an intranasal and intraocular irrigation process using a syringe (Nasaline®; Entpro, Sweden), washing the nasal cavities with 100 ml of saline and the eyes with 25 ml of saline. Then the washing solution from each subject was collected to investigate the number of pollen grains in it.

The subjects were subsequently exposed to moderate-concentrated JC pollen (4500 grains/m³) for 2 hours. Each subject recorded their symptoms (such as sneezing, nasal discharge, nasal obstruction, itchy nose, itchy eyes, and tears) into computers at certain points (after 15, 30, 60, 90, and 120 minutes from the start of exposure).

The symptoms were classified as follows: 1. none, 2. mild, 3. moderate, 4. severe, and 5. very severe. We collected tissue papers with which subjects had blown their noses and measured them by weight. The weight difference between tissue papers before and after use was considered to be the weight of nasal secretion.

The amount of intranasal and intraocular pollen was determined by the following methods. We added 1.25 *g* of Safranine-O (Wako Jyunnyaku Kogyo Inc. Tokyo) to 50 ml of saline and 50 ml of ethanol to create stain solutions, and 5 ml of this stain solution was added to the nasal or ocular lavage fluid. Furthermore, we added 20 drops of Proteinase K (Dako Cy-

Table 2 The number of intraocular and intranasal pollen grains in the first part of the study.

Subject No.#	1	2	3 *	4	5	6	7	8	9	10	Mean ± SD
Intraocular pollen	2	13	3	6	15	20	12	6	49	10	13.6 ± 13.6 (grains)
Intranasal pollen	175	175	303	160	198	522	90	260	281	328	249.2 ± 120.9 (grains)

One subject (*) exited the chamber 10 min earlier since she was about to sneeze.
The remaining subjects were able to stay for an hour without developing symptoms.
As to the subject numbers, see Table 1

Table 3 The number of subjects who showed nose or eye symptoms during 2-hour allergen exposure.

	15	30	60	90	120	(min)
Rhinorrhea	1	3	3	6	8	
Sneezing	0	1	1	4	4	
Nasal obstruction	1	4	6	6	7	
Nasal itching	2	4	4	7	8	
Eye itching	2	5	6	7	6	
Watery eyes	0	2	3	3	3	
Nasal secretion (g/hr)	←	0.23 ± 0.39	→	←	0.73 ± 0.83	→

There were increases in the number of subjects who developed nasal and ocular symptoms in a time dependent manner. Six subjects developed nasal obstruction after 60 minutes of exposure, of which 3 subjects marked their symptoms as moderate while 1 subject marked them as severe. Although 8 subjects developed rhinorrhea and nasal itching after 2 hours of exposure to the allergen, only 4 subjects developed sneezing and their symptoms were mild. We measured the amount of nasal secretion from 3 subjects in the first half of the study and from 7 in the latter half of the study. Ocular symptoms also developed as time went by. During the study period, 1 subject showed neither nasal nor ocular symptoms.

tomation Inc, Tokyo, Japan) to nasal lavage fluid in order to reduce its viscosity. After shaking the nasal or ocular lavage fluid for 5 minutes at room temperature, it was put into a suction filtration device using 0.5 μm filter papers (Advantee Cellulose Nitrate, Toyo Roshi Inc, Tokyo, Japan). Hereafter, the leftover solution was washed once more with saline and the solution was put into a suction filtration device to keep any single grain from being left behind. In this way, all the filter papers were examined under a light microscope (×100) and the pollen count was recorded.

RESULTS

The subjects consisted of 5 men and 5 women with mild symptoms. Their ages ranged from 32 to 48 years (the average age was 38.5 ± 5.4). Background factors of the subjects are shown in Table 1.

In the first part of this study, the temperature, the humidity, and the concentration of pollen grains in the room were kept within the targeted values. That is, the average number of pollen grains was 2572.8 ± 264.5 grains /m^3, the average temperature 22.1 ± 0.14℃, and the average humidity 44.7 ± 0.48% (Fig. 4). Intranasal pollen counts in the subjects ranged from 90 to 522 and intraocular grains ranged from 2 to 49. No relation was seen between the positions of the subjects within the chamber and the numbers of pollen detected in the nose and eyes. Even though 1 subject had to leave the chamber 10 minutes earlier than the scheduled time because of symptom development, the rest of the subjects were able to stay in the room for an hour without developing symptoms. The average number of intranasal pollen grains and intraocular grains was 249.2 ± 120.9 and 13.6 ± 13.6, respectively (Table 2).

In the second part of this study, the temperature, the humidity, and the concentration of pollen grains inside the chamber were within targeted values: the average number of pollen grains was 4367 ± 207 grains/m^3, the temperature 22.2 ± 0.35℃, and the humidity 44.8 ± 0.65% (Fig. 5). As time went by, more and more subjects started developing nasal symptoms such as nasal discharge, sneezing, nasal obstruction, and itchy nose. Only 3 subjects showed nasal discharge 60 minutes after the start of exposure, but 6 subjects after 90 minutes and 8 subjects after 120 minutes. Likewise, the number of subjects who developed nasal obstruction increased after 60 minutes of exposure, of which 3 subjects marked their symptoms as moderate while 1 subject marked severe. Although 8 subjects showed nasal itching after 120 minutes, only 4 subjects developed sneezing during exposure and their symptoms were mild. Three subjects blew their nose within 1 hour of the first half of the study, and 7 in the latter half. The amount of nasal secretion in the first half and the latter half of the study was 0.23 ± 0.39 g/hour and 0.73 ± 0.83 g/

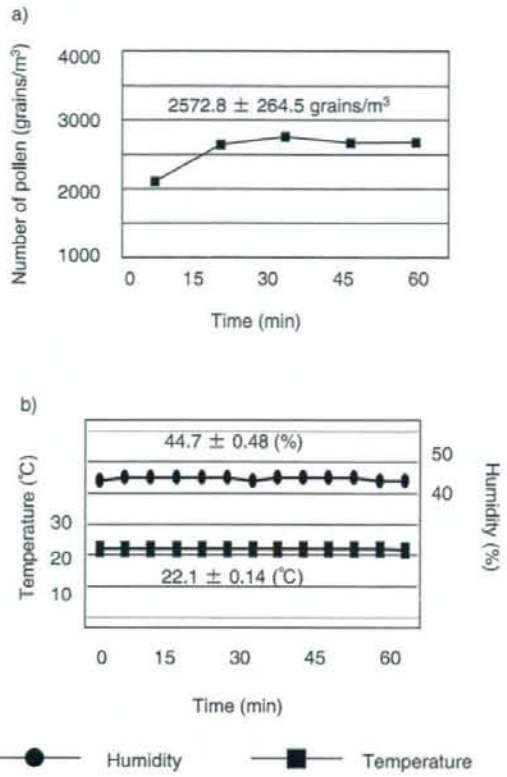


Fig. 4 The pollen count (**a**) and temperature and humidity (**b**) in the chamber during the first part of the study. The pollen count at each point shows the average value during 15 minutes. The average number of pollen grains was 2572.8 ± 264.5 grains/m³, the average temperature 22.1 ± 0.14℃, and the average humidity 44.7 ± 0.48%.

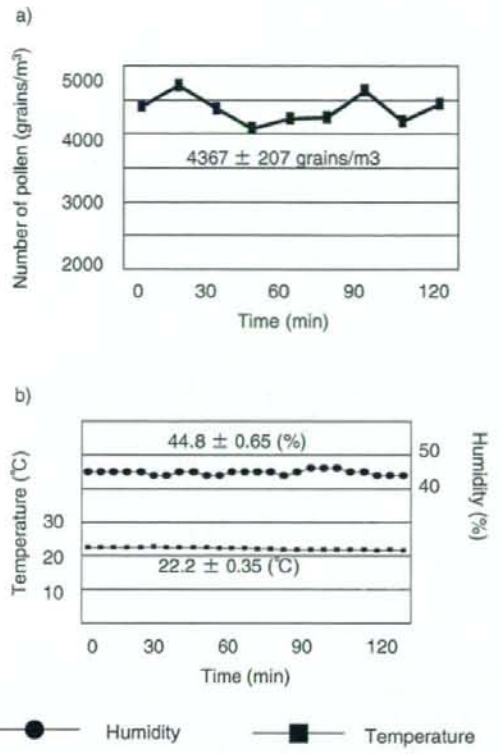


Fig. 5 The pollen count (**a**) and temperature and humidity (**b**) in the chamber during the second part of the study. The average number of pollen grains was 4367 ± 207 grains/m³, the average temperature 22.2 ± 0.35℃, and the average humidity 44.8 ± 0.65%.

hour respectively (Table 3).

With regard to ocular symptoms, half of the subjects developed itchy eyes within 30 minutes after entering the chamber, increasing to 7 subjects as time went by. Their symptoms, however, were mild. In addition, only 3 subjects developed epiphora while other 3 subjects experienced neither itchy eyes nor epiphora in the chamber. During the 2-hour exposure to pollen, 1 subject showed no nasal or ocular symptoms. No adverse event was observed in these studies. None of the subjects showed late nasal reaction after returning home.

DISCUSSION

This is a preliminary study showing the amount of intranasal or intraocular pollen as well as allergy symptoms in mildly symptomatic subjects with JC pollinosis under well controlled conditions in an OHIO chamber.

There are several allergen exposure units in Europe and the US[6-8] and a lot of useful data are available in the treatment of allergic rhinitis.[10,11] However it is difficult to simply compare the figures for pollen dispersal between the results obtained from each exposure unit. Each chamber uses a different method to count the pollen grains. For instance, our OHIO chamber uses KC-20 (Rion Co, Tokyo, Japan), a laser particle counter which measures the number of particles between 10 and 100 m in diameter. On the other hand, the Japanese Environment Ministry, which has been measuring pollen counts at various stations and providing information during pollen seasons, uses a different kind of laser-counter (KH-3000: Yamato Seisakujo, Japan). We attempted measurements with both KC-20 and KH-3000 and discovered that there is a correlation between the outcomes of the two methods of measurement. We also discovered that KC-20 yields values 5.9 times larger than those obtained using KH-3000 (data not shown).

In the first part of the study, subjects were exposed

to 2500 grains/m^3 of JC pollen, which is equivalent to the amount of airborne pollen grains in the early stages of the pollen season. Although their allergic symptoms were reported to be mild, one of the subjects had to leave the room before the scheduled time since the subject was about to sneeze. The other 9 subjects were able to remain in the room for an hour without developing any allergic symptoms. We found that the average number of intranasal and intraocular pollen grains was 249.2 and 13.6, respectively. The subjects sat still during the study for up to 1 hour and did not move around, so their eyes simply received pollen grains, and their shedding tears and blinking cleared the pollen grains. On the other hand, subjects actively inhaled pollen grains through their noses, allowing more pollen to precipitate in the nose and they did neither sneeze nor blow their noses. These could be the possible reason why the number of intranasal pollen grains was much larger than that of the intraocular pollen grains. Gotoh *et al.*[12] conducted a study on the ratio of intranasal to intraocular pollen numbers, which were obtained from healthy volunteers walking in the open air at an ordinary speed for half an hour. Their study showed the almost same result with ours.

In the second part of the study, the concentration of pollen dispersed was increased to 4500 grains/m^3. This concentration is equivalent to the amount of airborne pollen grains during the midterm and late stages of the pollen season. Nasal and ocular symptoms gradually developed in a time dependent manner, but these symptoms were mild.

Okuda *et al.*[13] measured the number of intranasal grains of JC pollinosis patients during pollen seasons over several years. They showed that the average number of JC pollen found in a patient's nose was about 20, although the amount of pollen varies every year. They concluded that 90 to 150 pollen grains were considered to be sufficient to cause symptoms from the dynamic study of pollen in the nose.

In this study, we found that 90 to 500 pollen grains in the nose were not enough to develop nasal symptoms. The only exception was the subject who exited the room in 50 minutes due to sneezing, whose intranasal pollen counted 303. There is a difference in the number of pollen which develops nasal symptoms observed in our study and in the study conducted in a natural environment.[13] The following could be the reason for the difference; subjects in this study were mildly symptomatic patients with JC pollen; subjects had not received repetitive exposure to JC pollen, because the study was conducted 3 months ahead of the pollen season; and subjects were under psychological pressure since they had never experienced an environmental exposure study.

This is the first study to show the intranasal and intraocular pollen grains and allergic symptoms using the OHIO Chamber. As far as pollinosis is concerned, however, our data cannot be immediately generalized since the results depend on the amount of pollen, the priming effects of the nasal mucosa, and the severity of the patients' symptoms. We need to evaluate the results of our data carefully. Therefore, further investigations are required to decide an appropriate amount of pollen and exposure time to obtain reproducible results and to secure the safety of the subjects.

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Upregulation of the Release of Granulocyte-Macrophage Colony-Stimulating Factor from Keratinocytes Stimulated with Cysteine Protease Activity of Recombinant Major Mite Allergens, Der f 1 and Der p 1

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Key Words
Keratinocytes · Cystatin A · Granulocyte-macrophage colony-stimulating factor · Recombinant allergens · House dust mite · Der f 1 · Der p 1 · Cysteine protease

Abstract
Background: Although exposure to mite allergens is an important risk factor for the production of IgE and is associated with various allergic diseases, there has been uncertainty as to the route of exposure by which sensitization occurs. Cystatin A is a skin-derived dominant inhibitor against proteolytic activity of major mite allergens, Der f 1 and Der p 1, and blocks the upregulation of IL-8 release from human keratinocytes stimulated with the allergens. We analyzed whether the stimulation of keratinocytes with the allergens upregulates the release of granulocyte-macrophage colony-stimulating factor (GM-CSF), which has many actions relevant to allergic diseases including atopic dermatitis, and if so, whether cystatin A can block this process. ***Methods:*** Normal human keratinocytes and the human keratinocyte cell line HaCaT were stimulated with recombinant group 1 allergens in the absence or presence of cystatin A. ***Results:*** Stimulation with the recombinant allergens upregulated the release of GM-CSF from normal human keratinocytes in a culture with high calcium concentration and HaCaT cells, which could be inhibited by the addition of cystatin A. The allergens exhibiting proteolytic activity did not digest cystatin A. Proteolytic activity of recombinant Der f 1 was partially regenerated after incubation with keratinocytes even without preactivation by L-cysteine. ***Conclusion:*** Proteolytic activity of recombinant Der f 1 and Der p 1 upregulates GM-CSF and IL-8 release from keratinocytes in vitro, suggesting possible contributions to sensitization through the skin and the perpetuation of atopic dermatitis, as well as a homeostatic role for cystatin A against inflammation of the skin.

Introduction

Exposure to house dust mite allergens is an important risk factor for the production of IgE and is associated with allergic diseases such as asthma, rhinitis and atopic dermatitis (AD) [1–3]. House dust mite group 1 allergens, Der f 1 from *Dermatophagoides farinae* and Der p 1 from *Dermatophagoides pteronyssinus*, are major allergens belonging to the papain-like cysteine protease family [4].

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Their proteolytic activity has been suggested to be involved in the pathogenesis of allergies by facilitating the passage of their own and other allergens across the epithelium and skin [5–7], cleaving and/or interacting with cell surface molecules and intrinsic protease inhibitors and modulating the functions of various cells [8–15]. The cysteine protease activity is inactivated by oxidation of the catalytic thiol group of mite group 1 allergens during storage or purification but can be regenerated by treatment with reducing reagents such as dithiothreitol (DTT), L-cysteine, glutathione and others. Although Herbert et al. [5] speculated that Der p 1 could be activated by reduction with glutathione present in airway tissue on the basis of their in vitro results, where and how cysteine protease activity of the mite group 1 allergens can be activated in vivo is unclear.

Using a yeast expression system, we succeeded in the preparation of recombinant forms of Der p 1 (rDer p1) and Der f 1 (rDer f 1), which are similar to their natural counterparts in allergenicity and show a similar substrate specificity for cysteine protease activity to the natural types [16–19]. They show cysteine but no serine protease activity, which was often reported to be detected in natural types purified from mite culture extract possibly due to contamination of mite-derived serine proteases [18]. Recombinant forms without yeast-derived hyperglycosylation exhibit similar molecular sizes and secondary structures to their natural types [17, 19]. Like the natural Der p 1, they also cleave human CD23, CD25 and α_1-antitrypsin and elicit IgE production in mice [11, 20, 21], and the stimulation of human keratinocytes upregulates the release of IL-8 [15]. The proteolytic activity of rDer f 1 with yeast-derived hyperglycosylation caused a reduction in the barrier function of the skin in nude mice [7].

Cystatin A, which is also called 'stefin A', is a skin-derived dominant inhibitor against the cysteine protease activity of Der p 1 and Der f 1 and blocks the upregulation of IL-8 release from keratinocytes stimulated with rDer p 1 and rDer f 1 [15]. Very recently, a significant association of a cystatin A genotype with AD has been reported [22]. Thus, a system including activation and suppression of keratinocyte response against mite cysteine proteases exists in the skin. Analysis of interactions between environmental and genetic factors such as those between protease allergens and their endogenous inhibitors is important to elucidate the pathogenesis of allergic diseases and to prevent their development [23–25].

In airway epithelial cells, cysteine protease activity of Der p 1 has been reported to upregulate the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) along with IL-8 [12]. GM-CSF has many actions relevant to allergic inflammation including AD [26, 27]. In this study, we analyzed whether the stimulation of keratinocytes with the proteolytic activity of rDer f 1 and rDer p 1 upregulates the release of GM-CSF, and if so, whether cystatin A can block this process.

Materials and Methods

Der f 1, Der p 1 and Cystatin A

rDer f 1 and rDer p 1 without yeast-derived hyperglycosylation (Der f 1-N53Q and Der p 1-N52Q) were prepared as described previously [17, 19, 28]. Recombinant human cystatin A was purchased from R&D Systems (Minneapolis, Minn., USA). Human sweat-derived cystatin A was purified as described [15].

Stimulation of Keratinocytes

Normal human keratinocytes (Cascade Biologics, Portland, Oreg., USA) were cultured in HuMedia-KG2 (0.15 mM $CaCl_2$) or EpiLife-KG2 (0.06 mM $CaCl_2$; Kurabo Industries, Osaka, Japan) supplemented with 0.1 ng/ml epidermal growth factor, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin B, and 0.4% vol/vol bovine brain pituitary extract. Cells of the human keratinocyte cell line HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, Mo., USA) supplemented with 10% FCS and antibiotics. They were stimulated with rDer f 1, rDer p 1 and an agonist peptide of the human protease-activated receptor 2 (PAR-2; Bachem, Bubendorf, Switzerland) in basal media without the supplements, as previously described [15]. The cells were incubated for 24 or 48 h, and the supernatants were collected and subjected to ELISA to measure released cytokines including GM-CSF and IL-8 using kits (DuoSet; R&D Systems).

Proteolytic Activity

Proteolytic activity was measured as described previously [18], with some modifications, as indicated in the figure legends. Time courses of digestion of a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA (Peptide Institute, Osaka, Japan), were monitored.

SDS-PAGE Analysis

The recombinant group 1 allergens were activated with L-cysteine as described previously [20]. Activated rDer f 1 and rDer p 1 at 400 nM were added to 100 nM human sweat cystatin A or 200 nM human α_1-antitrypsin as a positive control in reaction buffer composed of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. Unactivated rDer f 1 and rDer p 1 were used as controls. After incubation for 2 h at 37°C, the samples were subjected to SDS-PAGE.

Statistical Analysis

Group data were statistically analyzed with Prism version 4.0 (GraphPad, San Diego, Calif., USA). The unpaired Student's t test (two-tailed) or the Tukey post hoc test followed by a one-way ANOVA was used to evaluate the significance of differences. A p value <0.05 was regarded as statistically significant.

Results

Release of IL-8 from Keratinocytes Stimulated with rDer f 1

The dose and time dependency of the release of IL-8 from keratinocytes stimulated with activated rDer f 1 was analyzed (fig. 1). Cysteine protease activity of rDer f 1 was regenerated by pretreatment with L-cysteine. The release from HaCaT cells and primary cultured normal human keratinocytes was upregulated by 100 nM of rDer f 1 in the supernatants collected at 24 and 48 h after stimulation. The concentration of IL-8 released was higher at 48 than at 24 h.

Release of GM-CSF and IL-8 from Keratinocytes Stimulated with rDer p 1 and rDer f 1 and Its Inhibition by Cystatin A

Recombinant cystatin A dose-dependently inhibited the protease activity of rDer p 1 and rDer f 1 (fig. 2a). HaCaT cells released more GM-CSF and IL-8 on stimulation with activated rDer p 1 and rDer f 1 at 100 nM after 48 h than unstimulated cells, and the addition of an equimolar amount of cystatin A blocked this process (fig. 2b). At a low calcium concentration, the release of IL-8 from normal human keratinocytes was upregulated on stimulation with rDer p 1 and rDer f 1 at 100 or 200 nM at 48 h, inhibited by the addition of cystatin A in an equimolar amount, while the release of GM-CSF was not upregulated (fig. 2c). At a high calcium concentration, release of both GM-CSF and IL-8 from normal human keratinocytes was upregulated, blocked by the addition of cystatin A (fig. 2d). The release of GM-CSF upregulated by the stimulation with rDer p 1 and rDer f 1 was greater in normal human keratinocytes than in HaCaT cells (fig. 2b, d, left panels). The values of GM-CSF and IL-8 on stimulation with an agonist peptide of PAR-2 as a positive control were not affected by the addition of cystatin A, indicating the blocking effect by cystatin A to be specific to the proteolytic activity of rDer p 1 and rDer f 1.

Cystatin A Was Not Digested by the Proteolytic Activity of rDer p 1 and rDer f 1

The stability of cystatin A on exposure to the proteolytic activity of rDer p 1 and rDer f 1 was evaluated by SDS-PAGE. Sweat-derived cystatin A was stable even though it interacted with active rDer p 1 or rDer f 1 (fig. 3). Under the same conditions in the presence of L-cysteine, they cleaved α_1-antitrypsin, as reported previously [20].

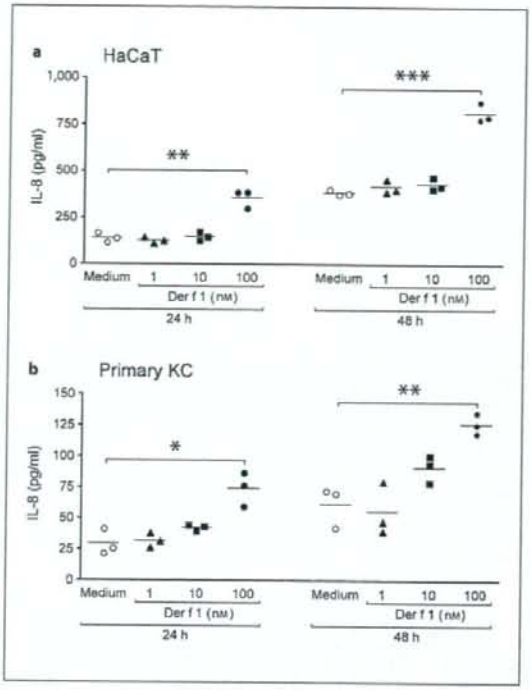


Fig. 1. Dose and time dependency of IL-8 release from keratinocytes stimulated with rDer f 1. **a** Human keratinocyte cell line HaCaT. **b** Primary cultured normal human keratinocytes (KC). Cysteine protease activity of rDer f 1 was activated with L-cysteine prior to stimulation of the cells. Basal media of DMEM and HuMedia-KG2 were used for the stimulation of HaCaT and normal human keratinocytes, respectively. The culture supernatants collected 24 or 48 h after the beginning of the stimulation were assayed. Each point corresponds to the value for each well. Results for 3 wells are shown. Bars indicate means. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control (medium) by unpaired t test (two-tailed).

rDer f 1 with or without Pretreatment with L-Cysteine Upregulated the Release of IL-8 and GM-CSF

Whether regeneration of cysteine protease activity by pretreatment with L-cysteine prior to the stimulation of keratinocytes is essential to the upregulation of the release of the proinflammatory cytokines was analyzed. With or without the pretreatment with L-cysteine, rDer f 1 upregulated the release of IL-8 (fig. 4) and GM-CSF (fig. 5c) from normal human keratinocytes.

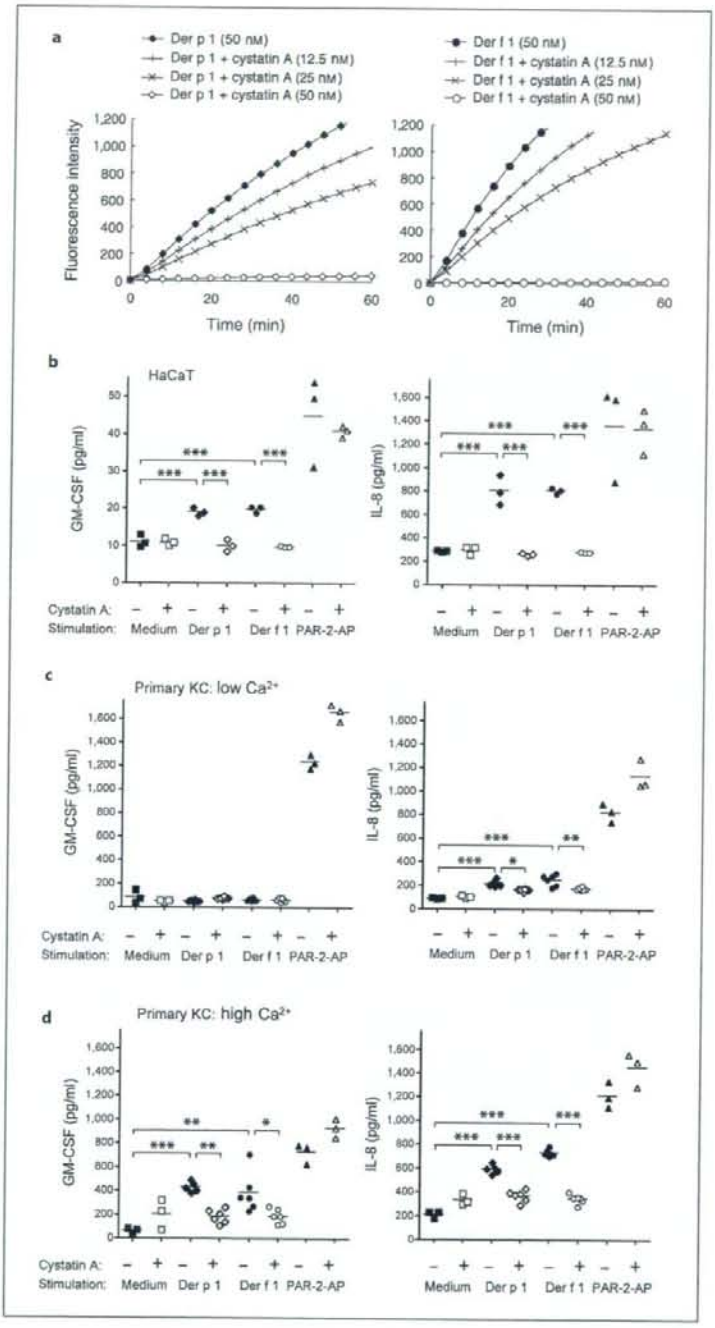


Fig. 2. Release of GM-CSF and IL-8 from keratinocytes stimulated with rDer p 1 and rDer f 1 and its inhibition by cystatin A. **a** Inhibition of proteolytic activity of rDer p 1 and rDer f 1 by recombinant cystatin A. rDer p 1 and rDer f 1 were activated with DTT, incubated with cystatin A and subjected to the assay in a reaction buffer composed of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. Time courses of digestion of a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA, were measured on a fluorometer (Fluoroskan Ascent; Labsystems, Vantaa, Finland). Final concentrations of the allergens, DTT and the substrate were 50 nM, 1 mM and 0.1 mM, respectively. Final concentrations of cystatin A are indicated (12.5, 25 and 50 nM). **b–d** Release of GM-CSF and IL-8 from HaCaT cells and primary cultured normal human keratinocytes (KC). rDer p 1 and rDer f 1 were activated with L-cysteine prior to stimulation of the cells. Basal medium of DMEM was used for the stimulation of HaCaT (**b**). Basal media of HuMedia-KG2 containing 0.15 mM (**c**) and 1.35 mM $CaCl_2$ (**d**) were used for the stimulation of normal keratinocytes. Final concentrations of the allergens and recombinant cystatin A were 100 or 200 nM. The culture supernatants collected 48 h after the beginning of stimulation were assayed. Each point corresponds to the value for each well. Results for 3 or 6 wells are shown. Bars indicate means. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between cells stimulated with allergens (Der p 1 or Der f 1) and those unstimulated (medium) and between the absence (–) and presence of cystatin A (+) by the Tukey post hoc test followed by one-way ANOVA (two-tailed). PAR-2-AP = Agonist peptide of PAR-2 (100 μM for HaCaT and 500 μM for normal keratinocytes).

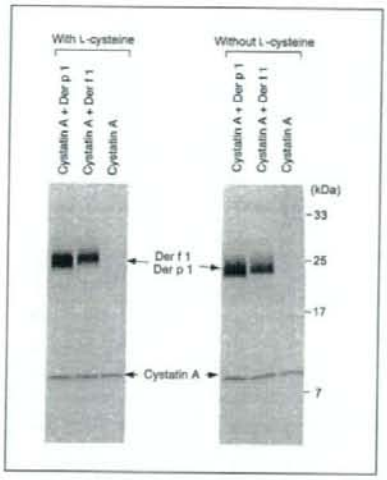


Fig. 3. Cystatin A was not digested by rDer p 1 and rDer f 1. The allergens were incubated in a buffer with or without L-cysteine prior to the reaction. Bands for rDer p 1, rDer f 1 and sweat-derived cystatin A are indicated by arrows. The gel after SDS-PAGE under nonreducing conditions was stained with silver (Silver Stain II kit; Wako, Osaka, Japan).

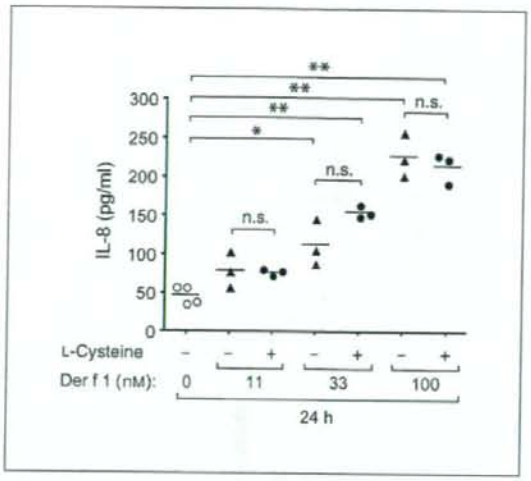


Fig. 4. rDer f 1 with or without preactivation with L-cysteine upregulated IL-8 release from primary cultured normal human keratinocytes. rDer f 1 were activated with L-cysteine (+) or unactivated (–) prior to stimulation of the cells. Basal medium of Hu-Media-KG2 was used for the stimulation. The culture supernatants collected 24 h after the beginning of the stimulation were assayed. Final concentrations of rDer f 1 were indicated. Each point corresponds to the value for each well. Results for 3 or 4 wells are shown. Bars indicate means. * $p < 0.01$ and ** $p < 0.001$ compared with the negative control by the Tukey post hoc test followed by one-way ANOVA. n.s. = Not significantly different ($p > 0.05$) between groups stimulated with rDer f 1 with and without pretreatment with L-cysteine compared by unpaired t test (two-tailed).

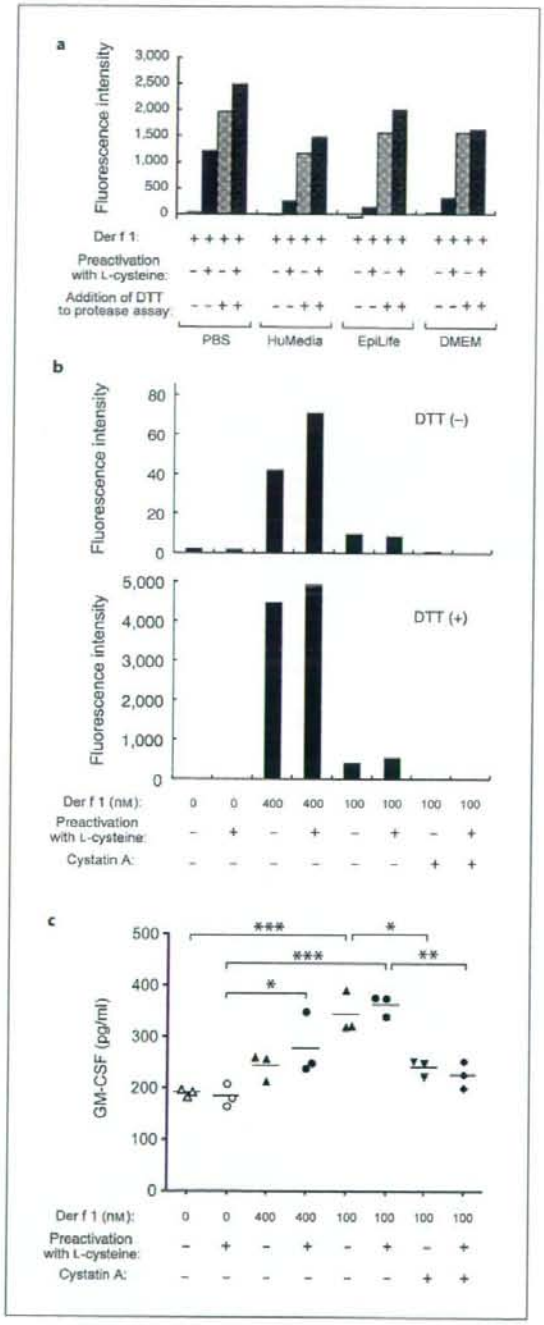
Proteolytic Activity of rDer f 1 in Culture Media before and after Incubation with Keratinocytes

Just after dilution in the basal media, unactivated rDer f 1 showed almost no proteolytic activity, and rDer f 1 activated with L-cysteine showed lower activity compared with rDer f 1, which was kept active by DTT during the protease assay (fig. 5a).

Culture supernatants recovered 36 h after the beginning of the stimulation of normal keratinocytes showed proteolytic activity (fig. 5b, upper panel), although the activity was lower (1/100 or less) compared with those kept active by DTT during the protease assay (fig. 5b, lower panel). The activity was similar between unactivated rDer f 1 and rDer f 1 activated with L-cysteine prior to the stimulation (fig. 5b, upper panel) and was eliminated by addition of fresh cystatin A in the protease assay (unpublished data). Keratinocytes were detached from the bottom of the culture plate at 400 nM of rDer f 1 with or without pretreatment with L-cysteine but not at 100 nM (unpublished data). Addition of cystatin A to the stimulation kept the proteolytic activity inactive (fig. 5b, upper panel, cystatin A+), even in the presence of DTT during the protease assay (fig.5b, lower panel, cystatin A+), and inhibited the upregulation of GM-CSF release (fig. 5c, cystatin A+).

Discussion

Although exposure to mite allergens is an important risk factor for the production of IgE and is associated with various allergic diseases, there has been uncertainty as to the route of exposure by which sensitization occurs. We demonstrated that stimulation of keratinocytes with the proteolytic activity of rDer f 1 and rDer p 1 upregulates the release of GM-CSF along with IL-8 (fig. 1, 2, 4, 5c), and cystatin A, which is the skin-derived dominant and stable inhibitor against the proteolytic activity of the allergens (fig. 2a, 3, 5b), can block this process (fig. 2, 5c). rDer f 1 with or without pretreatment with L-cysteine upregulated the release of proinflammatory cytokines (fig. 4, 5c), and cysteine protease activity of unactivated rDer f 1 was partially regenerated after incubation with keratinocytes (fig. 5b). These results suggest the importance of the proteolytic activity of mite group 1 allergens



in sensitization through the skin, a homeostatic role for cystatin A, and that the catalytic thiol group of the cysteine protease allergens, which was oxidized during storage or purification, could be regenerated by interaction with the cells or components in the culture supernatant.

GM-CSF has many actions relevant to allergic diseases such as AD. A recent hypothesis is that lesional upregulation of GM-CSF contributes to the maintenance of the chronic inflammatory process in AD primarily by enhancing the antigen-processing capacity of Langerhans and dendritic cells [26]. We have reported that the proteolytic activity of rDer f 1 with yeast-derived hyperglycosylation causes a reduction in barrier function of the skin in nude mice [7]. Taken together, the cysteine protease activity of the mite group 1 allergens might be involved in sensitization through the skin by reducing the barrier function of the skin and providing a portal of entry for allergens and irritants, and by subsequently stimulating keratinocytes to upregulate the release of GM-

Fig. 5. Proteolytic activity of rDer f 1 in the cell culture media before and after incubation with primary cultured normal human keratinocytes. **a** Proteolytic activity of rDer f 1 in fresh basal media. The reaction was started with diluting rDer f 1 unactivated or activated with 5 mM L-cysteine in phosphate-buffered saline (PBS, pH 7.4) or in the basal media (HuMedia-KG2, EpiLife-KG2 and DMEM) containing a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA, with or without DTT (1/10 dilution). Time courses of digestion of the substrate were measured on a fluorometer (SpectraMAX GeminiEM; Molecular Devices, Sunnyvale, Calif., USA). Fluorescence intensities at 60 min are shown. Final concentrations of rDer f 1, L-cysteine, DTT and the substrate in the protease assay were 100 nM, 0.5, 1 and 0.1 mM, respectively. **b** Proteolytic activity within culture supernatants 36 h after the beginning of the stimulation of normal keratinocytes. The cells were stimulated with rDer f 1 unactivated or activated with L-cysteine in the absence or presence of recombinant cystatin A. Basal medium of EpiLife-KG2 was used for the stimulation. The reaction was started with diluting culture supernatants in PBS containing the substrate with or without DTT (1/2 dilution). Fluorescence intensities at 60 min are shown. The vertical scale is different between the upper and lower panels. Concentrations of rDer f 1 in the stimulation are indicated (400 and 100 nM) and correspond to 200 and 50 nM in the protease assay, respectively. The concentration of cystatin A in the stimulation was 200 nM. Concentrations of DTT and the substrate in the protease assay were 1 and 0.05 mM, respectively. **c** GM-CSF release from normal keratinocytes. The culture supernatants collected 48 h after the beginning of the stimulation were assayed. Each point corresponds to the value for each well. Results for 3 wells are shown. Bars indicate means. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with controls by the Tukey post hoc test followed by one-way ANOVA.