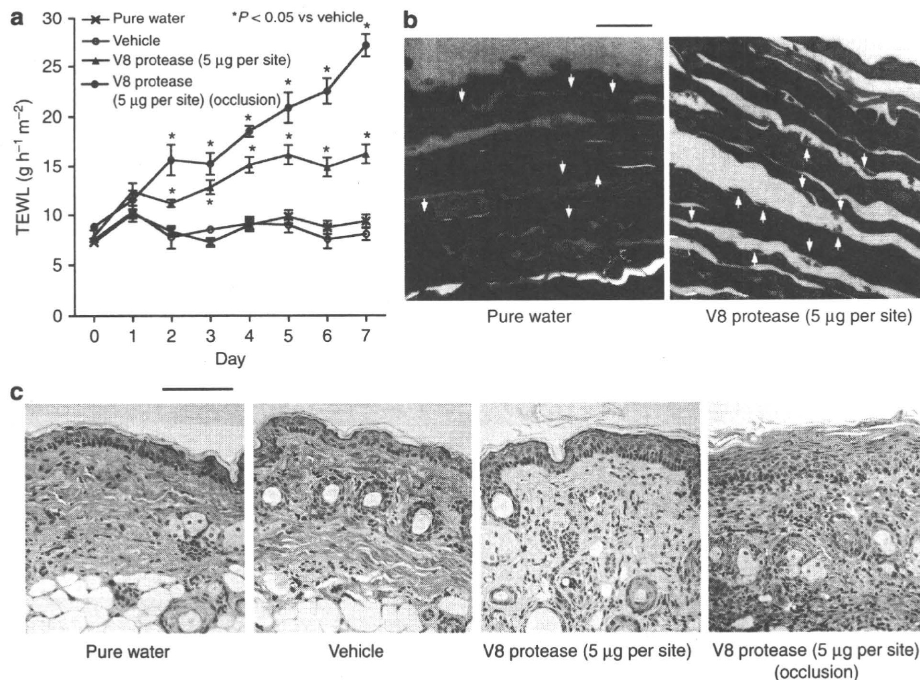

Figure 2. Effects of *Staphylococcus aureus* V8 protease application in hairless mice. Pure water, phosphate-buffered saline, or V8 protease ($5\,\mu g\,100\,\mu l^{-1}\,2\,cm^{-2}$ per site) was directly applied to the backs of 6-week-old female hairless mice (Skh:HR1 strain; Hoshino Laboratory Animals, Ibaraki, Japan) without a patch and occlusion, or with a patch containing V8 protease ($5\,\mu g\,100\,\mu l^{-1}\,2\,cm^{-2}$ per site) with occlusion using a polyurethane film was applied every day for 1 week. Transepidermal water loss (TEWL) was measured every day (**a**). After the last measurement of TEWL, specimens for electron microscopy and histology were prepared (**b** and **c**). (**a**) Epidermal permeability barrier dysfunction. Data represent the mean ± SEM for six mice per group. *$P<0.05$ compared with the control (vehicle) by one-way analysis of variance and Tukey's *post hoc* test was considered as significant. (**b**) Analysis of the stratum corneum (SC) by transmission electron microscopy. V8 protease induced structural disturbance of the SC, including loss of corneodesmosome integrity and loss of corneocyte cohesion. The arrows indicate corneodesmosomes. Bar = 1 μm. (**c**, histology) The tissue was fixed in formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin. V8 protease without occlusion induced an increase in epidermal thickness. With occlusion, it induced a greater increase in epidermal thickness and inflammatory infiltration in the dermis. Bar = 100 μm.

lesser extent after aspartate (Figure 1b). Epidermal permeability barrier dysfunction in nude mice was evaluated (Figure 1c and d) as previously reported (Nakamura *et al.*, 2006). A patch containing V8 protease with occlusion was applied to the backs of mice every other day for 1 week. Use of nude mice simplified the experimental procedure because of both the strain's hairlessness and the fact that the lack of a thymus excluded the effects of T-cell-mediated immunity. The critical epidermal permeability barrier is mediated by the outermost layer of the epidermis, the stratum corneum (SC). V8 protease induced the permeability barrier dysfunction, which was evaluated on the basis of two parameters: transepidermal water loss (Figure 1c) and penetration by riboflavin of the SC (Figure 1d) in a dose-dependent manner. Histology showed that V8 protease disturbed the structure of the SC but did not cause epidermal hyperproliferation and inflammatory infiltration (data not shown).

We next evaluated the effects of *S. aureus* V8 protease application, with or without occlusion, in hairless mice with a normally developed immune system (Figure 2). V8 protease with or without occlusion was applied every day for 1 week. Regardless of the presence of occlusion, application of V8 protease significantly increased transepidermal water loss compared with controls in a time-dependent manner (Figure 2a). Analysis of the SC by transmission electron microscopy revealed that the protease induced a structural disturbance of the SC without (Figure 2b, V8 protease) or with occlusion (data not shown). Histology revealed that the protease without occlusion induced an increase in epidermal thickness; with occlusion it induced not only a greater increase in epidermal thickness but also inflammatory infiltration in the dermis (Figure 2c). Analysis by scanning electron microscopy showed that a morphological change occurred on the skin surface of mice to which the protease was applied, also indicating that V8 protease made corneocytes less adhesive (data not shown).

S. aureus is not a normal member of the microflora colonizing the skin. In AD patients, however, *S. aureus* colonizes lesional skin, and colony counts even in nonlesional skin are often high (Aly *et al.*, 1977). The breakdown of the epidermal permeability barrier and the structural disturbance of the SC caused by the staphylococcal extracellular protease (Figure 1c and d and Figure 2) suggest that residential *S. aureus* could impair the epidermal barrier. Areas of the skin in which the barrier function is

impaired are likely to provide a portal of entry for various *S. aureus*-derived molecules, allergens such as house dust and pollen, and irritants that initiate and perpetuate cutaneous inflammation.

Interestingly, exfoliative toxins from *S. aureus* show sequence similarity to V8 protease and also exhibit a similar specificity of glutamate-specific cleavage (Dubin, 2002). Exfoliative toxins cause blisters in bullous impetigo and staphylococcal scaled-skin syndrome, and they hydrolyze a single peptide bond after glutamate 381 of desmoglein 1, a desmosomal cadherin that mediates cell–cell adhesion (Amagai *et al.*, 2000; Hanakawa *et al.*, 2002). Considering their similarity in structure and critical substrate specificity, we speculate that the staphylococcal glutamate-specific protease family, including exfoliative toxins and V8 protease, could abrogate the epidermal permeability barrier via desmoglein 1 cleavage in corneodesmosomes, which causes the loss of corneocyte cohesion and structural disturbance of the SC (Figure 2b). On the other hand, the highly specific preference for cleavage after glutamate (Figure 1b, LLE) suggests that the staphylococcal glutamate-specific protease family may not activate protease-activated receptor-2 (PAR-2) (Hachem *et al.*, 2006; Jeong *et al.*, 2008; Kato *et al.*, 2009) because the PAR-2-activating cleavage site is a peptide bond after arginine (Steinhoff *et al.*, 2005), although it may act in a cascade of activation of proteases and activate PAR-2 in a secondary manner.

In conclusion, our results imply the importance of staphylococcal proteases as an environmental factor that contributes to primary sensitization to allergens as well as to the vicious circle of *S. aureus* colonization and exacerbation of AD by abrogating the epidermal permeability barrier. As far as we know, this is the first demonstration of the epidermal barrier function being impaired by an *S. aureus* protease *in vivo*.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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CASE REPORT

A Case of Anaphylactic Reaction Following Matsutake Mushroom Ingestion: Demonstration of Histamine Release Reaction of Basophils

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ABSTRACT

Background: Matsutake mushroom is not recognized as a common food allergen. However, several case reports have suggested that this mushroom can induce anaphylaxis on rare occasions.

Case Summary: We report a woman with bronchial asthma, who experienced two episodes of Matsutake-induced anaphylaxis. Both the prick-to-prick test and basophil histamine release test showed positive reactions to this mushroom in this patient, but not in control subjects.

Discussion: Matsutake mushroom can, on rare occasions, cause anaphylaxis in sensitized people, a reaction so far observed only in Japan. Not ony the *in vivo* prick-to-prick test but also the *in vitro* basophil activation test utilizing the patient's blood represent useful methods for allergen identification and also for identification of sensitized subjects.

KEY WORDS

anaphylaxis, basophils, food allergy, histamine release, Matsutake mushroom

INTRODUCTION

Food allergy is a common disease, and its prevalence rate is reported to be as high as 2 to 4% in Japan and the United Status.[1,2] The symptoms related to food allergy often include eczema and digestive disorders; anaphylaxis requires special clinical attention because this reaction usually occurs unexpectedly and worsens very rapidly. Various foods are well known to be potential allergens capable of inducing anaphylaxis, but mushrooms are generally overlooked due to the rarity of allergic patients. Here, we report a patient with past anaphylactic episodes induced by Matsutake mushroom, confirmed by the skin test and basophil activation test.

CLINICAL SUMMARY

A 38-year-old woman with bronchial asthma told us, at a scheduled visit at the outpatient clinic of our hospital, that she had experienced immediate hypersensitivity reactions following Matsutake mushroom ingestion.

She developed atopic asthma at two years of age and has undergone specific immunotherapy with monthly subcutaneous injections of house dust extract since she was 23 years old. During childhood, she had no history of food allergy or eczema. At the age of 27, ingestion of a soup containing several kinds of vegetables and Matsutake mushroom was almost immediately—within ten minutes—followed by an anaphylactic reaction consisting of systemic urticaria, dyspnea and consciousness disturbance. She recov-

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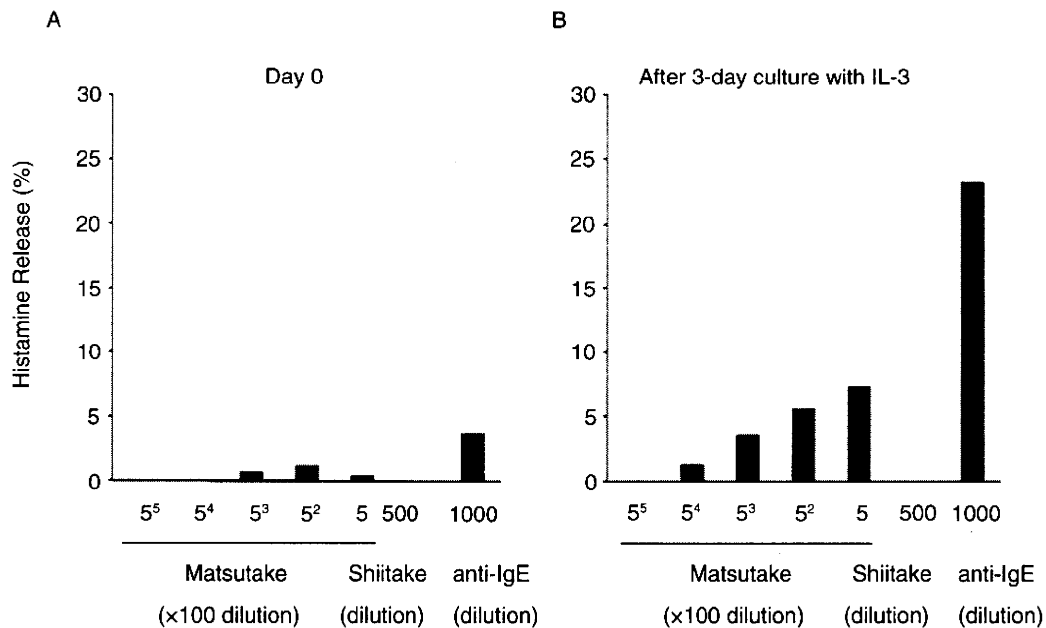

Fig. 1 Histamine release by the patient's basophils when exposed to Matsutake mushroom extract. Aqueous mushroom solutions were prepared by vigorous extraction of raw mushrooms in a 5-fold amount (weight/weight) of saline followed by clearing through 0.45-μm pore filters. Basophil preparations were assessed for degranulation immediately (**A**) or after 3-day culture with IL-3 at 300 pM (**B**). Cells were incubated with either polyclonal anti-IgE antibody, or Matsutake or Shiitake mushroom extract, and release of histamine was analyzed.[6] Data shown indicate the percentages of induced release over spontaneous release (less than 3% of total histamine). Mean values of duplicate determinations are shown. Fresh basophils showed nearly no release in response to anti-IgE antibody (**A**), indicating that the patient's basophils had a non-releasing phenotype.

ered from these symptoms after therapy in the emergency department of a local hospital. At that time, no clinical or laboratory analyses for allergen determination were performed. One year later, baked Matsutake again induced an anaphylactic reaction consisting of systemic urticaria and abdominal pain. Following the second episode of anaphylaxis, she surmised that Matsutake was the causal food. Since then, strict avoidance of this mushroom had prevented any further anaphylactic events. Interestingly, Shiitake and other mushrooms had never caused allergic reactions. Her serum IgE level was slightly elevated (178 IU/ml). Based on this clinical information, we presumed that Matsutake-induced immediate-type hypersensitivity reactions must have been the cause of her two anaphylactic episodes.

PATHOLOGICAL FINDINGS

Since a standardized extract solution of Matsutake mushroom for skin tests is not commercially available, we decided to perform a prick-to-prick test, using raw mushrooms according to previous case reports.[3,4] The patient showed a positive reaction in this test (wheal of 5 × 4 mm and flare of 26 × 15 mm at 15 minutes) for Matsutake mushroom, but was negative for Shiitake mushroom. On the other hand, ten healthy volunteers showed negative results to this test.

Next, a histamine release test was performed using the patient's basophils, after she granted informed consent. However, we found that her basophils had a non-releasing phenotype, lacking the ability to liberate histamine even when stimulated with optimal doses of IgE-crosslinking anti-IgE antibody (Fig. 1A). In the next experiment, her basophils were cultured with IL-3 at 300 pM for 3 days, as reported previously,[5] and then stimulated with various dilutions of extract solutions or with anti-IgE antibody. IL-3-cultured basophils demonstrated obvious release of histamine in response to anti-IgE antibody (Fig. 1B). In addition, the cultured basophils were sensitive to Matsutake mushroom extract: the cells released 7.2% of total histamine in response to 500-fold dilution of the extract. In parallel experiments, basophils were obtained from healthy volunteers, cultured and stimulated with Matsutake extract, but these cells never showed release of histamine.

Lastly, we assessed whether her serum contained Matsutake-specific IgE antibody capable of sensitizing basophils. Basophils from healthy volunteers were pretreated with IgE-stripping lactate buffer, pH 3.7, sensitized with the patient's serum for 2 hours at

37℃,[6,7] and then stimulated with mushroom (Matsutake and Shiitake) extracts. However, there was no apparent release of histamine from the sensitized cells (data not shown). We thus can only assume that Matsutake-specific IgE antibody in her serum must be low in either concentration or potency, and therefore incapable of inducing obvious sensitization *in vitro*.

DISCUSSION

Matsutake mushroom belongs to the Kishimeji family, and its scientific name is *Tricholoma matsutake*. This mushroom is available solely in the autumn season in Asian countries, and it is eaten after baking or boiling. Matsutake is quite expensive and thus much less often cooked in homes compared to other common mushrooms such as Shiitake. Anaphylaxis caused by Matsutake mushroom is rare: a total of only 13 cases have been reported to date,[3,4,8-14] all in Japan.

Our present patient told us that her two episodes of anaphylactic reaction might be clinically related to Matsutake mushroom, and that other mushrooms had never led to allergic symptoms. The prick-to-prick test was useful for allergen determination, consistent with other reports.[3,4] We further assessed the *in vitro* histamine release reaction using the patient's basophils and found that Matsutake mushroom extract induced degranulation of her basophils, but not of cells from nonallergic donors. These results clearly indicated that Matsutake was the causal food of her anaphylactic events. Thus, we strongly recommended that she should continue strict allergen (Matsutake) avoidance.

In the initial experiments using the patient's basophils, we found that they possessed a nonreleasing phenotype, i.e., they did not release histamine (<5% of total histamine) in response to optimal doses of anti-IgE antibody. After *in vitro* treatment by 3-day culture with IL-3, however, those nonreleasing basophils successfully converted to releasing cells, as reported in previous studies:[5,15] the cultured basophils demonstrated a histamine release reaction in response to a Matsutake extract. The precise antigenic molecules or epitopes in Matsutake mushroom extract have not been determined, but our *in vitro* findings and *in vivo* skin tests collectively suggest that her anaphylactic episodes were mediated by specific IgE and water-soluble antigen(s). We failed to induce passive sensitization of normal basophils with her serum, presumably due to low potency of the IgE. Other future cases showing a stronger basophil response would permit re-assessment of IgE involvement, allergen molecule determination and its precise heat-stability. Thus, *in vitro* analyses using basophil preparations are thought to be a safe and useful approach, capable of giving us insight into the pathogenesis of anaphylactic reactions.

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LETTER TO THE EDITOR

Dear Editor

Some asthmatics show elevation of the peripheral venous oxygen pressure (PvO_2)

Bronchial asthma is a common disease, characterized by reversible narrowing and hyperresponsiveness of the airways. Evaluation of airflow limitation and airway hypersensitivity is useful for objective assessment of asthma.[1,2] However, these tests may not be sufficient to fully elucidate the exact condition of asthma. During the course of many years of clinical experience, we noticed that some asthmatics present a contradiction by having a decent peak flow even while experiencing persistent dyspnea. We thus hypothesized that some other internal factor might be involved in the causation of some cases of dyspnea.

While searching for novel objective indices, approximately eight years ago we found marked elevation of the peripheral venous oxygen pressure (PvO_2) in an asthmatic subject. Further careful analysis led to a belief that said finding was reproducible and not erroneous, and was in fact occasionally seen in a small number of exacerbated asthmatics.[3-6] There were no signs of circulatory disturbances such as venous dilatation or peripheral temperature decline. Those deteriorating patients appeared to share several clinical features of not feeling well: lassitude, shortness of breath, palpitation and easy fatigability, combined with mild to moderate airflow limitation, and they each fulfilled the diagnostic criteria for asthma.[2] Besides having very high PvO_2 levels, they were intractable asthmatics who had responded poorly to a series of rescue medications and required hospitalization. Figure 1 plots the individual time-course data for PvO_2 obtained from 11 such patients without oxygen inhalation. Surprisingly, they initially showed an extraordinarily high PvO_2 (78.5 ± 16.9 Torr, n = 11) (median ± SD) (shown as the origin of Fig. 1), similar to that usually observed in arterial blood. The levels of $PvCO_2$ (38.8 ± 4.9 Torr, n = 11) also corresponded to the normal partial pressure of carbon dioxide in the arterial blood ($PaCO_2$). In clear contrast, the gas data for other severe, moderate or mild asthmatics, either stable or unstable, were within a narrow range and essentially the same as for healthy subjects (asthmatics without PvO_2 elevation: PvO_2 22.4 ± 7.3 Torr, $PvCO_2$ 48.6 ± 4.7 Torr, n = 36;

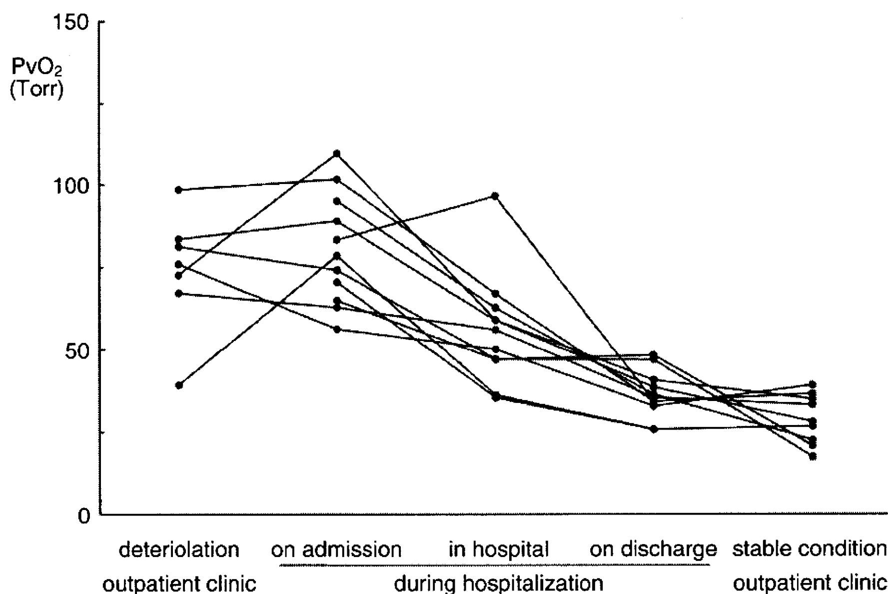

Fig. 1 Individual data for 11 consenting patients show that the PvO_2 levels were markedly elevated at the time of an emergency hospital visit and admission and gradually declined during therapy. All blood samples were obtained by venipuncture of the median cubital vein or forearm vein when blood cell and/or chemistry analysis was necessary. An aliquot of each sample was introduced directly into a gas analysis syringe and subjected to gas measurement within two minutes. The venous blood thus made no significant contact with room air, and contamination by arterial blood was unlikely. A pulse oxymeter indicated that O_2 saturation of the arterial blood was well maintained during venous blood sampling from the patients.

healthy: PvO_2 26.4 ± 10.2 Torr, $PvCO_2$ 47.8 ± 5.4 Torr, n = 26). As demonstrated in the figure, these very high PvO_2 levels gradually decreased, and inversely, $PvCO_2$ gradually increased (data not shown) during hospitalization, accompanied by alleviation of the dyspnea. Even high-dose systemic steroid therapy of these 11 patients failed to induce a rapid decrease in the PvO_2 level or rapid symptomatic relief. Oxygen inhalation often mildly alleviated their dyspnea. The most effective overall treatment for their symptoms, including fatigue, was rest.

We now believe that these gas analysis data clearly indicate the existence of a systemic mechanism driving PvO_2 elevation in some deteriorating asthmatics. Why and how their PvO_2 elevation occurs and is maintained, whether their airway inflammation is unique and the prevalence of such patients among asthmatics remain to be elucidated. Another important point is identification of a specific clinical biomarker in these patients that would be indicative of an elevated PvO_2 level during asthma exacerbation. At present, potentially useful clues for identifying these patients may be exertional breathlessness that seems out of proportion to the objective asthmatic state and a strangely bright red color of venous blood samples. Our preliminary analysis showed that the serum levels of lactate and pyruvate were elevated in these deteriorating patients, suggesting insufficient tissue oxygenation.[5] We presume that a high PvO_2 level translates into an inadequate oxygen supply and decreased consumption in peripheral tissues, leading to the lassitude, shortness of breath and easy fatigability seen in these deteriorating asthmatics.

The results shown herein were presented in part at academic meetings and have been published in abstract form.[3-6]

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