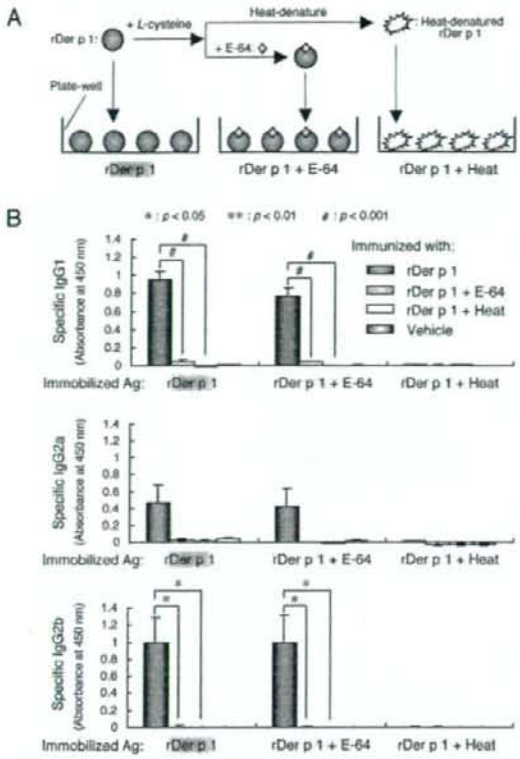

FIGURE 4. ELISA using plates coated with the immunized rDer p 1 derivatives used for immunization. *A*, Schematic procedure. The rDer p1, Der p 1-N52Q, was used for coating plates after the treatments shown. The rDer p 1 used for coating was not treated with L-cysteine. E-64-treated rDer p 1 and heat-denatured rDer p 1 used for coating were prepared by the same procedures for preparation of those for immunization. *B*, Levels of IgGs specific to the plate-coated Ags were measured in mouse sera at 4 wk. Serum dilution factors were 15,000, 100, and 100 for detecting Ag-specific IgG1, IgG2a, and IgG2b, respectively. The data shown represent the means ± SEM of the values for six mice except for the vehicle control (five mice). Student's *t* test (two-tailed) was used to evaluate the significance of the differences between treatments without and with E-64 or heating, and the statistically significant differences were indicated (*, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$).

relatively small reduction in Ab-binding affinity occurred with the E-64-modification (Fig. 5*A*, *upper panel*).

Differences in responses of splenocytes from mice immunized with the three types of rDer p 1

rDer p 1 stimulated the splenocytes recovered from rDer p 1-immunized mice to proliferate and produce IL-5 (Fig. 6, immunized with: rDer p 1) but did not stimulate those from E-64-treated rDer p 1-immunized mice (Fig. 6, immunized with: rDer p 1 + E-64), indicating that T cell differentiation during immunization is dependent on the proteolytic activity of the rDer p 1, as well as the production of the Abs. Splenocytes from heat-denatured rDer p 1-immunized mice exhibited the highest proliferation indexes and greatest production of IL-5 and IFN-γ (Fig. 6, immunized with: rDer p 1 + Heat). Thus, immunization with the three types of rDer p 1 induced distinctly different immune responses in terms of the Abs produced and cellular responses.

Discussion

We immunized mice with three types of rDer p 1 with functional or structural differences. The activated rDer p 1 has full cysteine protease activity and the tertiary structure of natural Der p 1 (16, 28, 43, 44), E-64-treated rDer p 1 is monomeric and similar in the global structure to rDer p 1 but enzymatically inactivated, and heat-denatured rDer p 1 is structurally disrupted and lacking enzymatic activity. Immunization with active rDer p 1 elicited particularly high levels of production of total IgE and Der p 1-specific IgG1 while that with inhibitor-treated rDer p 1 or heat-denatured rDer p 1 elicited much less production of total IgE and Ag-specific IgE and IgG Abs. The proliferative response of rDer p 1-restimulated spleen cells and production of IL-5 by the cells were significant on immunization with active rDer p 1. The cells from mice immunized with heat-denatured Der p 1 exhibited the highest levels of proliferation and production of IL-5 and IFN-γ. Thus, immunization with functional or structural derivatives of rDer p 1 induced distinctly different immune responses. To our surprise, E-64-treated rDer p 1 induced remarkably little production of both IgE and IgGs despite the maintenance of the global structure and conformational B cell epitopes. This finding is important because it strongly suggests that the IgE- and IgG-eliciting activity of rDer p 1 is dependent on its cysteine protease activity differing from the partial reduction in the IgE-eliciting activity of natural Der p 1 caused by E-64 treatment without affecting IgG production (40, 41).

Gough et al. (40) reported that E-64-treated natural Der p 1 adsorbed to Alum exhibited a partial reduction in IgE-eliciting activity (50–70% reduction of the absorbance in ELISA), and no reduction in Der p 1-specific IgG1 and IgG2b-eliciting activity compared with active Der p 1 while, in the present study, E-64-treated rDer p 1 elicited remarkably low levels of production of total IgE and Der p 1-specific IgE, IgG1, IgG2a, and IgG2b. They and we used the same mouse strain, CBA/J. The differences between our results and theirs might be attributable to differences in doses, timing, Ags used, strengths of the relative proteolytic activity (16, 28, 44) and purity of the Ags (16), or protocols for E-64 treatment. We emphasize that complete inactivation of the proteolytic activity is critical for elimination of the in vivo IgE-eliciting activity on the basis of our observations that E-64 treatment was not able to erase IgE-eliciting activity when the reactions were conducted with lower concentrations of E-64 and rDer p 1 (our unpublished observations), which is considered to decrease the probability of molecular collisions between E-64 and Der p 1 and to decelerate the speed of formation of E-64-rDer p 1 complexes. The cysteine protease activity of the house dust mite group 1 allergens has been reported to cleave some cell surface molecules (22–24, 26–28) and protease inhibitors (25, 26, 28, 30) and to modulate functions of various types of cells (21, 22, 27, 31–39). E-64-treated rDer p 1 with no proteolytic activity is considered to lose such activities and, in the present study, actually exhibited a remarkable reduction in Ab-eliciting activity. We conclude that the cysteine protease activity of the highly purified rDer p 1 is crucial for activity in vivo to elicit production of not only IgE but also IgGs differing from the partial and IgE-selective effects of natural Der p 1 suggested so far. We confirmed that the CBA/J strain used in the present study shows a highly sensitive response in terms of the production of total IgE compared with BALB/c and C57BL/6 against immunization with another recombinant mite group 1 allergen Der f 1 and that the Ab production is dependent on the proteolytic activity of the Ag in the three mouse strains (our unpublished observations).

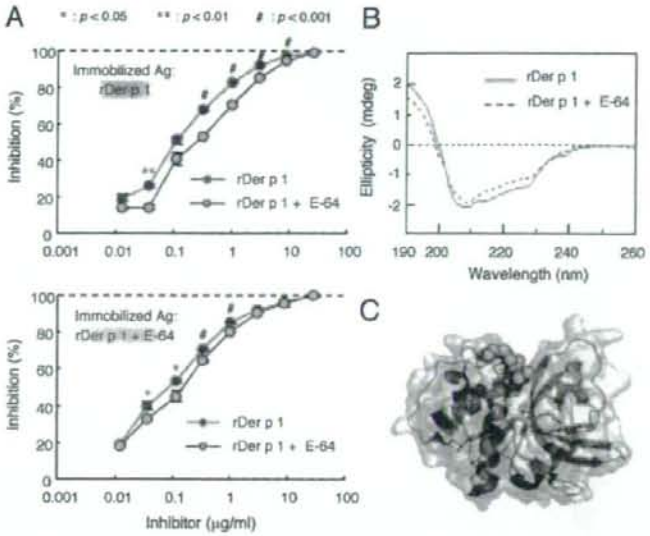

FIGURE 5. E-64-treated rDer p 1 retained the global structure. *A*, Inhibition assay (inhibition of IgG1-binding). A pooled serum, which was collected from the six mice immunized with activated rDer p 1 and diluted (1/20,000), was preincubated with serially diluted inhibitors (rDer p 1 or E-64-treated rDer p 1) and then added to plates, which were coated with rDer p 1, Der p 1-N52Q (*upper panel*), or E-64-treated rDer p 1 (*lower panel*). The percentage of inhibition was expressed as the relative reduction of the absorbance in each sample to that when no inhibitors were added. The data shown represent the means ± SEM of the values for the triplicate wells. Student's *t* test (two-tailed) was used to evaluate the significance of the differences between the two inhibitors, and the statistically significant differences were indicated (*, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$). *B*, Circular dichroism spectra. *C*, A model of the Der p 1/E-64 complex on the basis of the crystal structures of a mutant of pro-Der p 1 and a cathepsin K/E-64 complex. Transparent molecular surface and pink ribbon: Der p 1; yellow spheres: the catalytic residue Cys34 side chain; cyan spheres: E-64 that binds Cys34.

It appears that rDer p 1 has a greater effect on total IgE than OVA, and, by contrast, the specific IgE to Der p 1 based on the absorbance in ELISA was less than that to OVA. One possible explanation for the results is that the efficiency of the immobilization of Der p 1-WT could be lower than OVA. However, interestingly, the total IgE level peaked at 2 wk, preceding the elevation in the level of Der p 1-specific IgE. Therefore, other explanations also could be envisaged, such as the fact that the ratio total: specific IgE could be influenced by the nature of the administered protein, although whether the total IgE induced before elevation of Der p 1-specific IgE is truly nonspecific has not been clarified yet.

Our results clearly demonstrated that the tertiary architecture of the Der p 1 molecule is itself not enough to induce efficient production of both IgE and IgGs and that the proteolytic activity is crucial for eliciting positive immune responses in naive mice. This might imply that the commitment of the biochemical function of this molecule to the sensitization process makes this protein one of the most clinically relevant allergens. Although whether the biochemical functions of other major allergens have such a critical commitment to sensitization is an issue unsolved as yet (62, 63), we consider that studies based on this question might be important in elucidation of the pathogenesis of allergic diseases, prevention of their development, and therapy (50, 64, 65). Interestingly, people who do not become allergic to house dust mites do not make IgG specific for major house dust mite allergens (62, 63, 66, 67). The close link between allergenicity and immunogenicity of the major mite allergens in humans is similar to our results in mice where substantial increases in IgE, IgGs, and T cell responses were observed on immunization with active rDer p 1 but not on immunization with E-64-treated rDer p 1. Immune responses in people nonallergic to mites might be prohibited by physical and biochemical barrier systems, including intrinsic protease inhibitors produced at interfaces between the body and environment (29, 35), and/or might be actively regulated to achieve immunological tolerance against major allergens. The latter possibility that active regulation works in mice immunized with variants without the cysteine protease activity but retaining the tertiary global structure should be examined in a future study.

Heat-denatured rDer p 1, which is structurally disrupted and lacking enzymatic activity, elicited almost no production of IgE and Ag-specific Abs similar to E-64-treated rDer p 1. However, spleen cells from mice immunized with heat-denatured rDer p 1 proliferated more rapidly and produced much higher levels of cytokines than cells from active rDer p 1-immunized mice in response to restimulation with correctly folded rDer p 1, indicating that the heat-denatured rDer p 1 was not ignored by the immune system and Der p 1-specific T cells developed during the immunization. Disruption or modification of the tertiary structure of allergens is an effective way to reduce the IgE-binding activity of allergens when designing safer vaccines for allergen-specific immunotherapy (17, 47–50), and the change in the tertiary structure can modulate the immune response, suggesting that such modified allergens might contribute to the efficacy of the therapy (68–70). Recently, Magi et al. (69) reported immunogenic differences between a recombinant pro-Der p 1, which has an additional prodomain of 80 aa at the N-terminal of mature Der p 1 of 222 aa, and its heat-denatured form in mice. As they immunized mice with pro-Der p 1 or heat-denatured pro-Der p 1 and used pro-Der p 1 for the measurement of Ag-specific Abs and restimulation of spleen cells, the humoral and cellular responses potentially include responses against the prodomain, which composes approximately one-fourth of pro-Der p 1. In the present study, we immunized mice with mature rDer p 1 derivatives and evaluated humoral and

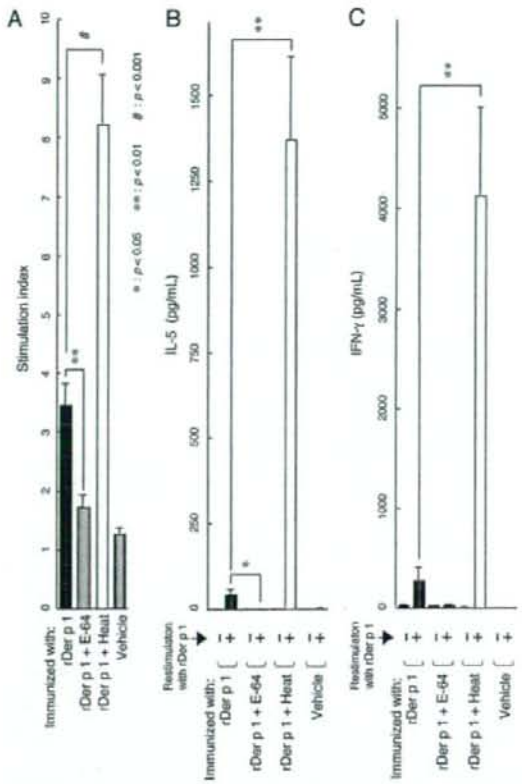

FIGURE 6. Splenocyte responses. Splenocytes recovered from mice immunized with active rDer p 1, E-64-treated rDer p 1, heat-denatured rDer p 1, or vehicle were restimulated with or without rDer p 1. *A*, Stimulation indexes of proliferation. *B*, IL-5 production. *C*, IFN-γ production. The data shown represent the means ± SEM of the values for six mice except for the vehicle control (five mice). Student's *t* test (two-tailed) was used to evaluate the significance of the differences between treatments without and with E-64 or heating, and the statistically significant differences were indicated (*, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$).

cellular responses using mature rDer p 1. Such rDer p 1 derivatives, which contain the mature sequence only, might have an advantage over the pro-Der p 1 derivatives in not eliciting the production of Abs or cellular responses against the prodomain.

In the case of Der p 1, the biochemical function of the cysteine protease activity also could be considered a target for therapy when designing a novel allergen-vaccine, along with the allergen's structure (17, 47–49). Interestingly, it was reported that a cysteine protease of the nematode *Nippostrongylus brasiliensis* elicited IgE and IgG1 responses in infected rats (71) and that IgE and IL-5 responses against a recombinant *Leishmania mexicana* cysteine protease, CPB2.8 (72), and IgG1 and IL-5 responses against highly purified papain, a potent occupational allergen (73), were eliminated or reduced by E-64 treatment of the Ags in mice as well as rDer p 1 in the present study. These results suggest that a common mechanism might determine the immune response to the cysteine proteases of allergens or parasite Ags. Our in vivo experimental system using the highly purified recombinant mite cysteine protease-allergens, which retain a structure and function similar to the natural counterparts (16, 28, 43, 44), will be useful for exploration of the mechanism involved and evaluation of recombinant allergen-vaccines for allergen-specific immunotherapy.

In summary, we investigated effects of E-64 treatment or heat denaturing of a highly purified and enzymatically active rDer p 1 on immune responses in vivo. The results suggest that the cysteine protease activity of rDer p 1 crucially contributes to in vivo immune responses, including the production of not only IgE but also IgGs against the tertiary architecture of this most clinically relevant allergen. Additionally, we showed that when functionally or structurally modified, rDer p 1 induced distinctly different immune responses. Our system would be useful for analysis of the mechanism determining whether sensitization or tolerance to major mite allergens develops in humans and that underlying the cysteine protease-dependent positive immune responses, and for evaluation of the potential of functional or structural derivatives as allergen-vaccines for specific immunotherapy. Further analysis of prophylactic or therapeutic approaches through the administration of functional or structural derivatives may contribute to immunotherapy for IgE/Th2-mediated allergic diseases.

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Disclosures

T. Takai, inventor, and the Chemo-Sero-Therapeutic Research Institute have a pending patent with the title "Modification of Group 1 Mite Allergen."

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precisely determined thus far, whereas the worldwide prevalence of EPP has been reported to be 1:75,000–1:200,000 (Todd, 1994). Thus, a nationwide survey and genotyping of the large number of Japanese EPP families is recommended and would be required to elucidate the virtual penetrance and prevalence of EPP in Japan.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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The study protocol was approved by the Ethics Committee of Hirosaki University Hospital. Informed consent was obtained from the patients described in this paper. This study was conducted according to the Declaration of Helsinki Principles.

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Reduction of Skin Barrier Function by Proteolytic Activity of a Recombinant House Dust Mite Major Allergen Der f 1

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TO THE EDITOR

Exposure to house dust mite allergens is an important risk factor for the production of specific IgE and is associated with allergic diseases such as asthma, rhinitis, and atopic dermatitis (Platts-Mills and Chapman, 1987). House dust mite Group 1 allergens, Der f 1 from *Dermatophagoides farinae* and Der p 1 from *Dermatophagoides pteronyssinus,* are major allergens and belong to the papain-like cysteine protease family (Thomas *et al.*, 2002). 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, cleaving and/or interacting with cell-surface molecules and intrinsic protease inhibitors, and modulating the function of various cells (Comoy *et al.*, 1998; Shakib *et al.*, 1998; Gough *et al.*, 1999; Takai *et al.*, 2005a). Although mite-derived proteolytic activities have been reported to disrupt the bronchial epithelial barrier (Herbert *et al.*, 1995; Wan *et al.*, 1999), whether they disrupt the skin barrier, which is considered a much more rigid barrier system, has not been investigated. Here, we test whether the proteolytic activity of Der f 1 causes a reduction in the barrier function of the skin in nude mice using a recombinant Der f 1 (rDer f 1) with full cysteine protease activity.

We demonstrated that rDer f 1 activated with L-cysteine reduced the barrier function of the skin in dose- and time-dependent manners (Figure 1) and that the reduction was dependent on its proteolytic activity (Figure 2). All animal studies have been approved by the Review Board of Juntendo University. By the use of nude mice, experimental procedures were simplified because of their hairless phenotype, and effects of T cell-mediated acquired immunity on the barrier dysfunction could be ignored. The critical permeability function of the skin is mediated by the outermost layer of the epidermis, the stratum corneum (Strid and Strobel, 2005). The barrier function was evaluated based on two parameters, transepidermal water loss (TEWL) and the penetration by riboflavin of the stratum corneum. TEWL is a parameter for dryness of the skin, whereas riboflavin penetration is considered a parameter of the accessibility of the skin to environmental allergens and irritants. On Day 7, TEWL and riboflavin penetration were significantly increased by administration of 1 or 5 μg/site (Figure 1a) and 5 μg/site (Figure 1b) of activated rDer f 1, respectively, compared with the vehicle control. No increases were observed on Day 3 (Figure 1c and d). TEWL was greater at the patched site than water-treated site even in the vehicle control suggesting that the vehicle solution containing diluted

Abbreviations: rDer f 1, recombinant Der f 1; TEWL, transepidermal water loss

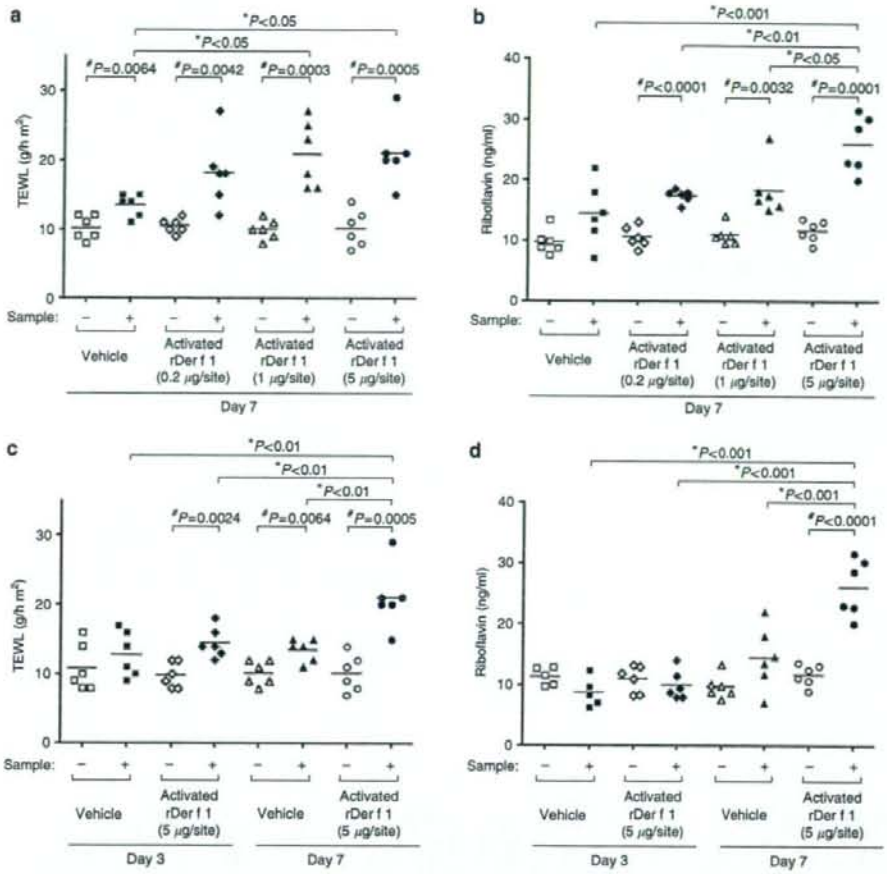

Figure 1. Dose- and time-dependent barrier dysfunction in the skin of nude mice at the sites where activated rDer f 1 was applied. Five-week-old female nude mice with the BALB/c background (Charles River Japan Inc., Yokohama, Japan) were maintained in a specific pathogen-free animal facility at Juntendo University. The glycosylated rDer f 1, designated Der f 1-WT, was expressed in yeast and prepared as described previously (Takai *et al.*, 2002, 2005b). The backs of mice anesthetized were lightly wiped with dry absorbent cotton once and cotton soaked with hexane twice. The use of hexane is to remove excess of scales. A rectangular piece of filter paper 1 cm × 2 cm (3MM Chr, Whatmann, Middlesex, UK) was placed on the back, and 100 μl of solution containing rDer f 1 was perfused to the paper. The rDer f 1 was preincubated with 1.4 mM L-cysteine and diluted with phosphate-buffered saline and pure water. The final concentrations of rDer f 1 and L-cysteine were 50, 10, or 2 μg/ml (5, 1, or 0.2 μg/100 μl/site) and 50 μM, respectively, and the final dilution of phosphate-buffered saline was 13/100. The paper was wrapped with poly(vinylidene chloride) film cut to a size of 2 cm × 3 cm and then immobilized with an adhesive bandage (KINO SELF; Nitto Denko Co., Osaka, Japan). Solutions containing rDer f 1 activated with L-cysteine or the vehicle only was applied to mice (+). Another site towards the tail away from the sample-loaded site was loaded with pure water (−) and treated as like the sample-loaded site. The solutions for loading were administered a total of four times (Day 0, 2, 4, and 6) or twice (Day 0 and 2) onto the patch every other day. The (**a** and **b**) dose and (**c** and **d**) time influenced the levels of the barrier dysfunction. (**a** and **c**) TEWL. On the day after the last administration (Day 7 or 3), the bandage, Saran Wrap, and paper were removed and the back of each mouse was lightly wiped once with absorbent cotton soaked with hexane, and then TEWL was measured with a Mobile Tewameter (Courage + Khazaka electronic GmbH, Köln, Germany). (**b** and **d**) Riboflavin test. After the measurement of TEWL, the amount of riboflavin to penetrate the stratum corneum was measured as described previously (Okuda *et al.*, 2002) with some modifications as follows. Cotton soaked with 100 μg/ml of riboflavin was placed on the site for 1 min and then removed, and the excess residual solution at the site was soaked up with wringed cotton. After drying for approximately 30 minutes, the stratum corneum was stripped with adhesive cellophane tape. The stripping was performed six times with the tape changed each time. The pieces of tape containing the stripped stratum corneum were dipped in 3 ml of a 1% sodium dodecyl sulfate solution overnight at 4°C protected from any light. The next day, the riboflavin was extracted from the tape by sonication. After vigorous mixing, the solutions were passed through a 0.2-μm filter and moved to new tubes. The concentration of riboflavin was determined by measuring the fluorescence of riboflavin. The data shown represent the values for six or five mice. Bars indicate means. The Tukey's *post hoc* test followed by one-way analysis of variance was used to evaluate the significance of the differences among the sites treated with the samples (+) (*$P<0.05$). Unpaired Student's *t* test (two-tailed) was used to evaluate the significance of the differences between the sites treated with each sample (+) and pure water (−) (#$P< 0.05$). A value of $P<0.05$ was regarded as statistically significant.

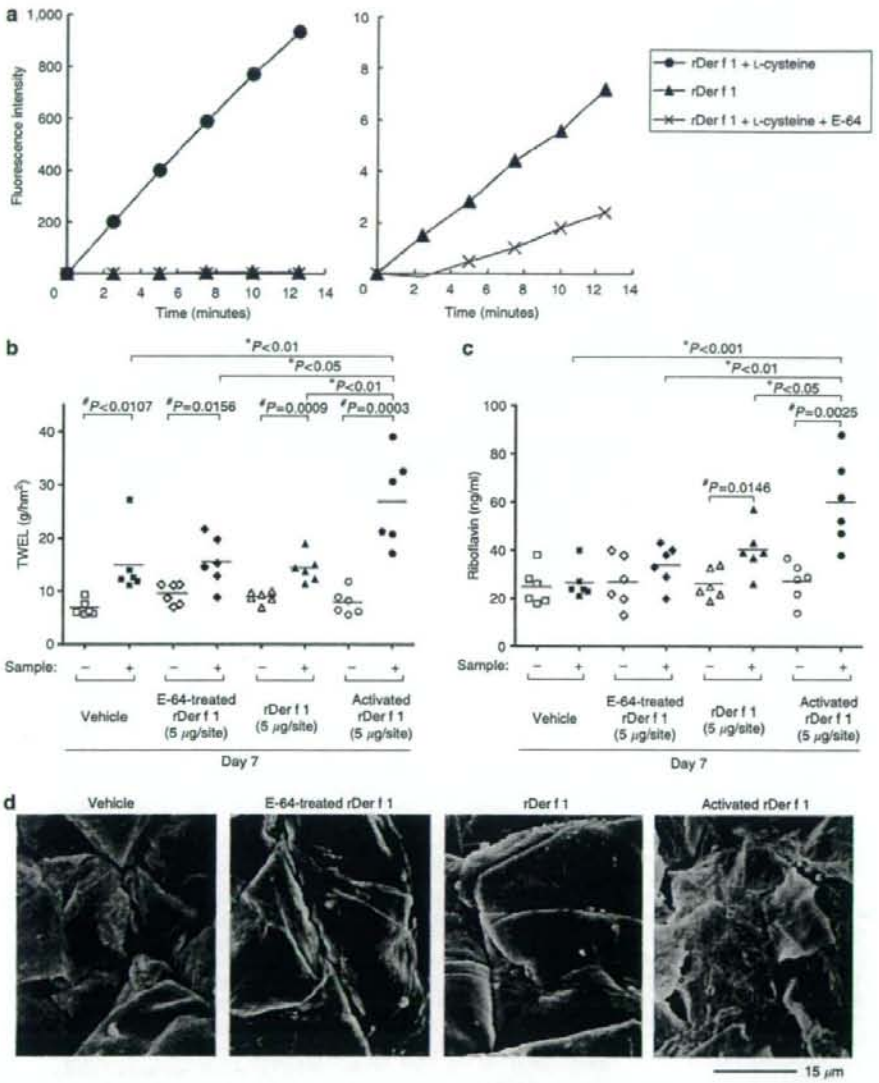

Figure 2. Barrier dysfunction and morphological changes in the skin of nude mice are dependent on the cysteine protease activity of rDer f 1. (**a**) Proteolytic activity of rDer f 1 before and after activation with L-cysteine, or after inactivation with an irreversible cysteine protease-specific inhibitor, E-64. The cysteine protease activity of rDer f 1 was measured as reported previously (Takai *et al.*, 2005c) except L-cysteine was used for the activation. Butyloxycarbonyl-Gln-Ala-Arg-MCA was used as the substrate. The scale of the horizontal axis in the right panel is 1/100 of that in the left panel. The activity of rDer f 1 in the absence of L-cysteine and in the presence of E-64 was, respectively, approximately 1/100 and 1/300 that of the fully activated rDer f 1. (**b** and **c**) Barrier dysfunction. To minimize the residual activity, E-64-treated rDer f 1 was prepared for administration to the mice by conducting the reaction with higher concentrations of rDer f 1 and E-64 and then dialyzing against phosphate-buffered saline. A solution containing rDer f 1 treated with L-cysteine (*Activated rDer f 1*), rDer f 1 not treated with L-cysteine (*rDer f 1*), a solution containing rDer f 1 treated with E-64 after its activation and then dialyzed against phosphate-buffered saline (*E-64-treated rDer f 1*) (5 μg/100 μl/site), or the vehicle only was applied to mice (+). Another site was loaded with pure water (−). The solutions for loading were administered four times onto the patch every other day (Day 0, 2, 4, and 6). On the day after the last administration (Day 7), measurement of TEWL (**b**) and riboflavin test (**c**) were performed. The data shown represent the values for six mice. The statistical analyses performed are as described in the legend of Figure 1. Data shown are representative of three independent patch experiments. (**d**) Scanning electron microscopy. Skin biopsies of the patched sites in mice not subjected to TEWL measurement and riboflavin test were analyzed.

phosphate-buffered saline and L-cysteine causes some disruption of the barrier as a background effect on Day 7 (Figure 1a and 2b). Although activated rDer f 1 significantly increased TEWL and riboflavin penetration and induced morphological changes at the sites where it was applied, rDer f 1 treated with an irreversible cysteine protease-specific inhibitor, E-64, did not (Figure 2b–d). Riboflavin penetration was greater at the sites exposed not only to activated rDer f 1 but also to unactivated rDer f 1 than at the water-treated sites (Figure 2c), indicating that the weak activity of the unactivated rDer f 1, which was not treated with L-cysteine, (Figure 2a) or the activity partially regenerated under the conditions on the patched skin was enough to cause minor barrier disruption.

An understanding of the mechanism of primary sensitization to allergens is important in elucidating the pathogenesis of allergic diseases and for possibly preventing their development. Mite allergens are an important factor in atopic dermatitis (Tan *et al.*, 1996; Friedmann, 1999; Kramer *et al.*, 2006), and Der f 1 and Der p 1 are present on the human skin (Yasueda *et al.*, 2003). Fecal pellets of *D. pteronyssinus* are known to contain Der p 1 at relatively high concentrations (10 mg/ml) (Tovey *et al.*, 1981) together with other mite serine proteases (Thomas *et al.*, 2002). Although it is difficult to relate the concentrations used in the present study to the amounts of mite proteases present on the human skin, we speculate that the amount of Der f 1 or Der p 1 at each point on the human skin, where mite fecal pellets directly attach, locally reaches to the amount applied in our experiments (four times administrations of 5 μg/100 μl/2 cm^2/site = 20 μg/2 cm^2). The barrier dysfunction and morphological changes caused by the cysteine protease activity of rDer f 1 suggest that repeated exposure to mite bodies and fecal pellets could disrupt the local skin's barrier function. Areas of the skin where the barrier function is reduced are likely to provide a portal of entry for allergens and irritants that both initiate and perpetuate cutaneous inflammation. Recently, we have found that unglycosylated rDer f 1 and rDer p 1 activates cultured human keratinocytes, and a skin-derived cysteine protease-specific inhibitor, cystatin A, can block the activation (Kato *et al.*, 2005) and that their proteolytic activity is crucial for IgE and IgG responses in mice (Takai *et al.*, unpublished observation; Kikuchi *et al.*, 2006). Taken together, the cysteine protease activity of the mite major group 1 allergens is now considered involved in the sensitization process in at least three ways, by reducing the barrier function of the skin and enabling allergens to penetrate, by stimulating keratinocytes, and by modulating immune responses. As no apparent epidermal proliferation and infiltration were induced on Day 7 in the present study using nude mice (unpublished observation), we consider that the barrier dysfunction was induced independently of inflammation. The process from the initial barrier disruption by mite proteases to inflammation with histological and immunological changes in atopic dermatitis is an important issue to be investigated in future studies.

As far as we know, this is the first demonstration of the barrier function of the skin being disrupted by the proteolytic activity of an allergen *in vivo*. Dry skin and an impaired barrier function are hallmarks of the pathogenesis of atopic dermatitis (Ogawa and Yoshiike, 1993; Leung *et al.*, 2004). Combination of barrier disturbance by a genetically determined barrier defect (Palmer *et al.*, 2006) and barrier disruption by some environmental allergens might increase the risk of sensitization to allergens toward IgE production and contribute to exacerbations of atopic dermatitis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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UV Increases the Nuclear Localization of Apurinic/Apyrimidinic Endonuclease/Redox Effector Factor-1 in Human Skin

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TO THE EDITOR

The apurinic-apyrimidinic endonuclease/redox effector factor-1 (APE/Ref-1) is a ubiquitous and bifunctional protein, which has both an apurinic-apyrimidinic endonuclease DNA repair activity and a nuclear redox activity (Tell *et al.*, 2000a; Yang *et al.*, 2005). Those two activities are split into two functionally independent domains of the protein: the N-terminus is principally devoted to the redox activity, whereas the C-terminus exerts enzymatic activity on abasic sites of DNA (Tell *et al.*, 2005). Recently, it was reported that overexpression of APE/Ref-1 protects melanoma cells from cisplatin or from H_2O_2-induced apoptosis, whereas an increased rate of apoptosis is observed in the presence of APE/Ref-1 antisense. There are several previous reports detailing the subcellular localization of APE/Ref-1, which is translocated into the nuclei after exposure of human cells to oxidative stress (Tell *et al.*, 2000b). Variable nuclear/cytoplasmic distributions of this protein are observed in different human tissues (Freitas *et al.*, 2003; Tanner *et al.*, 2004). APE/Ref-1 has also been shown to control p53 activity through redox alteration. p53 is induced in cultured human skin fibroblasts after treatment with H_2O_2, an oxidant produced in cells during exposure to solar UV radiation (Vile, 1997). APE/Ref-1 is also linked to apoptosis, associated with thioredoxin. The thioredoxin reductase/thioredoxin system has been shown to translocate into the nucleus both in keratinocytes and in melanocytes (Hirota *et al.*, 1999; Schallreuter *et al.*, 2006). Therefore, the nuclear *versus* cytoplasmic distribution of APE/Ref-1 may be regulated by the environment.

We investigated the effects of UV on human skin of various pigmented phenotypes before and at different times after a single minimal erythemal dose UV exposure. This study involved human subjects representing different racial/ethnic groups, and details of the protocols, subjects of the study, and UV doses have been published previously (Tadokoro *et al.*, 2003, 2005; Yamaguchi *et al.*, 2006). This study was approved by the Food and Drug Administration (FDA) Research Involving Human Subjects Committee, and adhered to the Helsinki Guidelines; informed consent was obtained from each of the study subjects. A bank of FS lamps (National Biological Corp., Twinsburg, OH) was used as the source of UV radiation (60% UVA and 40% UVB). The relationship between UV exposure and apoptosis prompted us to investigate the expression of APE/Ref-1 (and its subcellular distribution) in human skin before and after UV exposure.

Immunohistochemical staining and quantitation of images was carried out as detailed previously (Tadokoro *et al.*, 2003, 2005; Yamaguchi *et al.*, 2006). Mouse anti-human APE/Ref-1 antibody (Affinity BioReagents, Golden, CO) was used at 4°C at a 1:100 dilution. Alexa and 4, 6-diamidino-2-phenylindole (DAPI) fluorescence was observed and analyzed using a Leica DMRB/DMLD laser microscope (Leica Microsystems,

Abbreviations: APE/Ref-1, apurinic-apyrimidinic endonuclease/redox effector factor-1; DAPI, 4, 6-diamidino-2-phenylindole

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Application of Immunoreaction Enhancer Solutions to an Enzyme-Linked Immunosorbent Assay for Antigen-Specific IgE in Mice Immunized with Recombinant Major Mite Allergens or Ovalbumin

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Key Words
Enzyme-linked immunosorbent assay · Allergen-specific IgE · Immunoreaction enhancer solutions · Protein G · Recombinant mite allergens · Der p 1 · Der f 1 · Der f 2 · Ovalbumin

Abstract
Background: Weak signals for allergen-specific IgE are a problem in murine models for the study of allergies. It has been reported that the removal of IgG from murine sera enhances signal intensity. Very recently, buffer solutions designed to enhance signals in immunoassays have been developed and made commercially available. ***Methods:*** Sera from mice immunized either with a recombinant form of one of the major mite allergens Der p 1, Der f 1 and Der f 2, or with ovalbumin adsorbed to alum were used for the assays. Total IgE was measured by a sandwich enzyme-linked immunosorbent assay (ELISA). Allergen-specific IgE was assayed using plates coated with the allergens after the removal of IgG from sera with protein G-coupled sepharose beads in wells of other plates or with the use of commercially available enhancer solutions without the removal of IgG. IgE binding was detected with horseradish peroxidase-conjugated anti-murine IgE monoclonal antibody as the secondary antibody. ***Results:*** Significant levels of total IgE were produced after the immunizations. The in-well pretreatment of diluted sera (1/10 dilution) with protein G-coupled beads enhanced the signals for allergen-specific IgE. The use of the enhancer solutions for dilution of the sera and secondary antibody and prolonged incubation remarkably enhanced the signals at a more extensive dilution of sera (1/200 or less) without the removal of IgG. ***Conclusions:*** An ELISA simply modified with the use of immunoreaction enhancer solutions has advantages in terms of signal intensity and ease of handling for the detection of allergen-specific murine IgE and would be useful for the study of allergies with murine models.

Introduction

Immediate hypersensitivity is caused by the release of mediators from mast cells and basophils activated through aggregation of a high-affinity IgE receptor (FcεRI) at the cell surface and allergen-specific IgE, cross-linked by multivalent allergens derived from house dust mites, pollen and so on [1, 2]. Clinically, elevated

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levels of total or allergen-specific IgE in serum are a major manifestation of allergic diseases. For the estimation of allergen-specific IgE levels in sera, an enzyme immunoassay using a solid phase such as a cellulose sponge and paper disc to which an excess of allergen molecules can be immobilized has been used.

In mice immunized with model allergens such as ovalbumin (OVA) or clinically relevant allergens, the signal intensity for allergen-specific IgE in an enzyme-linked immunosorbent assay (ELISA) using plastic plates coated with the allergens is low, although total IgE can be easily detected with a sandwich ELISA using a set of 2 anti-murine IgE monoclonal antibodies (mAbs). The low signal intensity for allergen-specific IgE is mainly due to allergen-specific IgG, which is present in the sera from immunized mice and competitively inhibits the binding of allergen-specific IgE to the immobilized allergen. It has been reported that the removal of IgG from murine sera by passage through a packed protein G-coupled column [3] or by preincubation with protein G-coupled magnetic beads in tubes [4] enhances signal intensity. However, the loading of sera onto the column needs a relatively large volume and the process is time-consuming. Although the use of biotinylated allergens for the detection of allergen-specific IgE trapped on plates coated with anti-IgE antibody (Ab) is another way to improve signal intensity, the possibility that the modification disrupts some IgE epitopes cannot be excluded.

Very recently, buffer solutions designed to enhance signals in immunoassays have become commercially available [5–7]. In the present study, we evaluated the effects of in-well pretreatment of sera with protein G-coupled sepharose beads and the use of enhancer solutions on signals for the binding of allergen-specific IgE to plates coated with recombinant forms of the clinically relevant major house dust mite allergens [8–11] Der p 1, Der f 1 [12–17] and Der f 2 [18–20], or with OVA. We demonstrate that both methods are effective and that especially the latter has remarkable advantages in terms of signal intensity and handling.

Materials and Methods

Recombinant Der p 1, Der f 1 and Der f 2

Recombinant mature forms of Der p 1 and Der f 1 without (Der p 1-N52Q and Der f 1-N53Q) and with yeast-derived hyperglycosylation (Der p 1-WT and Der f 1-WT) were prepared as previously described [12, 13, 16]. Der p 1-N52Q (rDer p 1) and Der f 1-N53Q (rDer f 1) were used for immunization after incubation with L-cysteine [15]. As the B cell epitopes for recombinant forms with and without hyperglycosylation are considered to be equivalent [12, 13], we used the hyperglycosylated Der p 1-WT and Der f 1-WT for detecting Der p 1- and Der f 1-specific Abs as alternatives to the unglycosylated rDer p 1 and rDer f 1, whose production is much lower than that of the hyperglycosylated forms in yeast [16]. Recombinant Der f 2 (rDer f 2) was prepared as previously described [18] and used for immunization and the coating of microtiter plates for ELISA for Der f 2-specific Abs.

Immunization of Mice

Six- to 8-week-old female CBA/J, BALB/c and C57BL/6 mice were purchased from Charles River Japan Inc. (Yokohama, Japan). The mice were immunized with rDer p 1, rDer f 1, rDer f 2 or OVA (albumin from chicken egg white, grade V; Sigma, St. Louis, Mo., USA) as previously described [15], with minor modifications as follows. They were given 4 (CBA/J) or 5 (BALB/c and C57BL/6) weekly intraperitoneal injections with 0.5–10 μg of the allergens adsorbed on alum (Imject Alum; Pierce Biotechnology Inc., Rockford, Ill., USA). Sera collected 1 week after the last injections were stored at –20°C before assays. Sera used for the experiment in figure 5 were collected after the mice were challenged intranasally twice with rDer p 1 (10 μg/head) or buffer alone 2 months after the last intraperitoneal injection.

Measurement of Total IgE

The total concentration of IgE (total IgE) in the sera was measured by sandwich ELISA using a set of 2 anti-murine IgE mAbs with purified murine IgE as the standard as described previously [15].

Allergen Immobilization and Blocking

Allergens (Der p 1-WT, Der f 1-WT, rDer f 2 and OVA) were diluted with phosphate-buffered saline (PBS) and 96-well microtiter plates for ELISA (MaxiSorp; Nunc, Roskilde, Denmark) were incubated with the allergen solutions (10 μg/ml, 50 μl/well) overnight at 4°C. After 1 wash with PBS containing 0.05% (v/v) Tween 20 (PBST), the plates were incubated with BlockAce (Snow Brand, Sapporo, Japan) diluted with pure water (1/4 dilution, 200 μl/well) for 1.5 h at 37°C or 3 h at room temperature. After 2 washes with PBST, the plates were incubated with sera.

Detection of Allergen-Specific IgE by ELISA after In-Well Pretreatment of Sera with Protein G-Coupled Sepharose Beads

Sera diluted with PBST containing 10% (v/v) BlockAce were depleted of IgG with protein G sepharose 4FF (Amersham Biosciences, Uppsala, Sweden) within wells of round-bottomed microtiter plates (Corning Inc., Corning, N.Y., USA) with agitation at a speed of 1,000 rpm by a bio-shaker (Titec, Saitama, Japan) for 30 min at room temperature. The total volume of the suspension was 78 μl/well, the volume of protein G beads 18 μl/well, the volume of original serum added was 6 μl/well and the final serum dilution was 1/10. After centrifugation with a swing rotor at 1,900 *g* for 2 min, the supernatants were used for assays. With a multichannel micropipette, volumes of 50 μl from the supernatants in the wells were carefully moved to wells on other plates, which were coated with allergens and blocked as described above. After incubation for 1–2 h at 37°C and 3 washes with PBST, horseradish peroxidase (HRP)-conjugated rat anti-murine IgE mAb

(clone LO-ME-2; Technopharm Biotechnology, Paris, France) diluted with PBST containing 10% (v/v) BlockAce was added to the plates (1/2,000 dilution, 50 μl/well). After incubation for 1–2 h at 37°C and 3 washes with PBST, the color was developed using tetramethylbenzidine as the substrate (100 μl/well) and the reaction was stopped by adding 2 N sulfuric acid (Wako, Osaka, Japan) (50 μl/well) as previously described [15]. The optical density at 450 nm, from which that at 570 nm was subtracted, was used as the signal for allergen-specific IgE.

Detection of Allergen-Specific IgE by ELISA with the Enhancer Solutions

Volumes of 50 μl of sera diluted with solution 1 of Can Get Signal (Toyobo, Osaka, Japan), one of the commercially available immunoreaction enhancer solutions, were added to the wells on the plates which were coated with allergens and blocked as described above. After incubation for 15 h at 4°C and 3 washes with PBST, HRP-conjugated anti-murine IgE mAb (clone LO-ME-2) diluted with solution 2 of Can Get Signal was added to the plates (1/2,000 dilution, 50 μl/well). After incubation for 5 h at room temperature and 3 washes with PBST, the color was developed for 20 min using tetramethylbenzidine (BD OptEIA kit; BD Biosciences, San Jose, Calif., USA) (100 μl/well) and the reaction was stopped by adding 2 N sulfuric acid (50 μl/well). The optical density at 450 nm, from which that at 570 nm was subtracted, was used as the signal for allergen-specific IgE.

Detection of Allergen-Specific IgGs by ELISA with the Enhancer Solutions

Allergen-specific IgGs were detected in the same way as allergen-specific IgE with the modification that incubation with the diluted sera and the secondary Abs was performed for 90 min at 37°C. The secondary Abs were HRP-conjugated anti-murine IgG1 mAb (clone X56; BD Biosciences), HRP-conjugated goat anti-murine IgG2a Ab (Southern Biotechnology Associates, Birmingham, Ala., USA) and HRP-conjugated rabbit anti-murine IgG2b Ab (Zymed, San Francisco, Calif., USA), which were all used at a dilution of 1/2,000.

Removal of IgE from Serum

Anti-murine IgE mAb (clone R35-72; BD Biosciences) was covalently coupled to CNBr-activated sepharose 4B beads (Amersham Biosciences) (250 μg of mAb/approximately 250 μl of beads) and blocked with 2-aminoethanol according to the manufacturer's instructions. The beads (approximately 250 μl) were suspended with PBS containing 0.01% NaN_3 (total volume of the suspension: 1 ml). The anti-IgE beads (10 μl of the suspension) were added to a PBS-diluted pooled serum (1/10 dilution, 180 μl) from 3 mice immunized with rDer p 1 and gently agitated by rotation overnight at 4°C. The supernatant was recovered and depletion with the fresh anti-IgE beads was repeated totally 3 times. The supernatant after the third treatment with the beads was used for ELISA. Control beads, to which no protein was coupled, were also prepared by blocking of the active sites on CNBr-activated sepharose 4B beads with 2-aminoethanol.

Statistical Analysis

Group data were statistically analyzed with Prism version 4.0 (GraphPad, San Diego, Calif., USA). The unpaired Student t test (two-tailed) or the Tukey post hoc test followed by one-way ANOVA were used to evaluate the significance of differences. $p < 0.05$ was regarded as statistically significant.

Results

In-Well Pretreatment of the Sera with Protein G-Coupled Sepharose Beads Enhanced the Signals for Allergen-Specific IgE

In this experiment, immunizing CBA/J mice 4 times with rDer f 1 activated with L-cysteine (0.5 μg/head), rDer f 2 (0.5 μg/head) or OVA (2.5 μg/head) resulted in elevated levels of total IgE in the sera (fig. 1a). Using 1/10-diluted sera pretreated with protein G-coupled sepharose beads (fig. 1b), a significant strengthening of signals for the allergen-specific IgE was obtained (fig. 1c). Without the pretreatment with protein G beads, the signals were much weaker or not detected at all (unpubl. data). Although the total concentration of IgE on immunization with rDer f 1 or rDer f 2 was higher than or similar to that on immunization with OVA (fig. 1a), the signals for Der f 1- or Der f 2-specific IgE were weaker than those for OVA-specific IgE (fig. 1c).

Use of the Enhancer Solutions Enhanced the Signals for Allergen-Specific IgE without Removal of IgG

In this experiment, immunizing CBA/J mice 4 times with activated rDer p 1 (2.5 μg/head) or OVA (2.5 μg/head) resulted in elevated levels of total IgE in the sera (fig. 2a). The effect of the commercially available enhancer solutions on the detection of allergen-specific IgE was evaluated. We determined conditions suitable for the detection of allergen-specific IgE signals (fig. 2b), including a prolonged incubation with the sera and secondary Ab and a more extensive dilution of sera, but not the removal of IgG with protein G beads. Surprisingly, even at dilutions of 1/1,000 and 1/5,000, respectively, Der p 1- and OVA-specific IgE signals were of significant intensity (fig. 2d, e). Although the total concentration of IgE was higher on rDer p 1 immunization than OVA immunization (fig. 2a), the titer evaluated from the titration curves for Der p 1-specific IgE was lower than that for OVA-specific IgE (fig. 1d, e).

For the detection of allergen-specific IgGs, prolonged incubation was not needed (fig. 2c). IgG1 showed the highest titer among the 3 subclasses tested, IgG1, IgG2a and IgG2b (fig. 2f, g). Without the use of the enhancer solutions, signals for allergen-specific IgGs were detectable, although less dilution was necessary to obtain the same signal levels (unpubl. data).

ELISA Using the Enhancer Solutions Exhibited Specificity to Immunized Allergens and High Sensitivity for Detection

In this experiment, immunizing CBA/J mice 4 times with activated rDer f 1 (0.5 μg/head), rDer f 2 (0.5 μg/head) or OVA (1 μg/head) increased the total concentration of IgE in the sera (fig. 3a). In the ELISA system using the enhancer solutions (fig. 2b), IgE from mice immunized with rDer f 1 bound to the plates coated with Der f 1-WT but not to the plates coated with rDer f 2 or OVA (fig. 3b). Similarly, IgE from mice immunized with rDer f 2 or OVA selectively bound to the plates coated with rDer f 2 (fig. 3b) or OVA (fig. 3b), respectively. With the same procedure (fig. 2b) – except that a conventional buffer, PBST containing the blocking reagent, was used for dilution of the sera and secondary Ab – the intensity of signals for the allergen-specific IgE was remarkably decreased, although specificity to the allergen was retained (fig. 3c). Although the total concentration of IgE on immunization with rDer f 1 or rDer f 2 was higher than o r s imilar t o t hat o n i mmunization w ith O VA (fig. 3a), the signals for Der f 1- or Der f 2-specific IgE were weaker than those for OVA-specific IgE (fig. 3b, c).

ELISA Using the Enhancer Solutions Could Effectively Detect Allergen-Specific IgE in Other Strains of Mice

In this experiment, immunizing BALB/c and C57BL/6 mice 5 times with activated rDer f 1 (10 μg/head) resulted in a rise and no change, respectively, in the total concentration of IgE relative to the vehicle control group (fig. 4a). However, in the ELISA system using the enhancer solutions (fig. 2b), significant levels of Der f 1-specific IgE were detected in the rDer f 1-immunized groups in both strains (fig. 4b).

Confirmation of Isotype Specificity to IgE and Evaluation of Nonspecific Binding of IgE to the Plate

In this experiment, CBA/J mice were immunized 4 times with activated rDer p 1 (2.5 μg/head). Two months later, the mice were intranasally challenged twice with rDer p 1 (10 μg/head). The sera from the 3 immunized mice were mixed as a pooled serum and used for the assays. Total IgE concentrations of the pooled serum from rDer p 1-immunized mice and from the vehicle control were determined as 3.5 and 0.11 μg/ml, respectively, by a sandwich ELISA. After the removal of IgE by treatment with anti-IgE beads, the titer for Der p 1-specific IgE was decreased to 1/125–1/25 compared with the serum treated with the control beads (fig. 5a) but the signals for Der p 1-specific IgGs were unchanged (fig. 5b), demonstrating an isotype specificity to IgE. The signals detected at dilutions of 1/40 and 1/200 (fig. 5a) are considered due to residual Der p 1-specific IgE, because less than 1% of total IgE remained after the treatment with anti-IgE beads (unpubl. data).

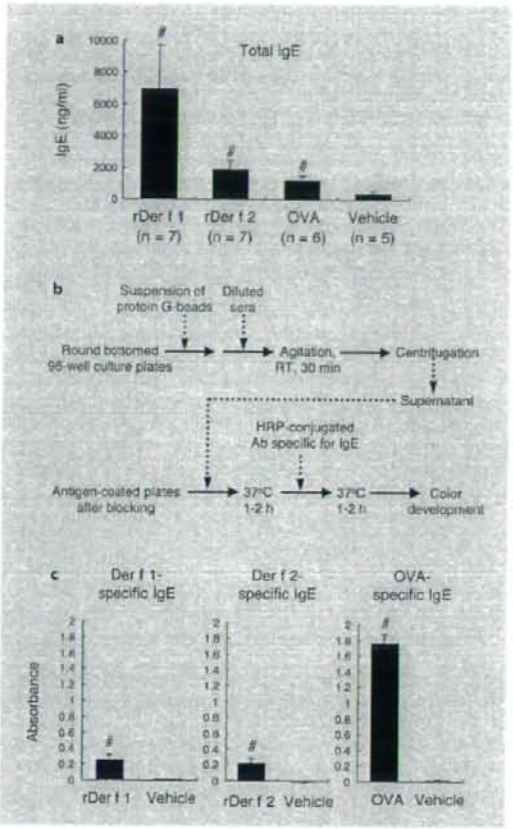
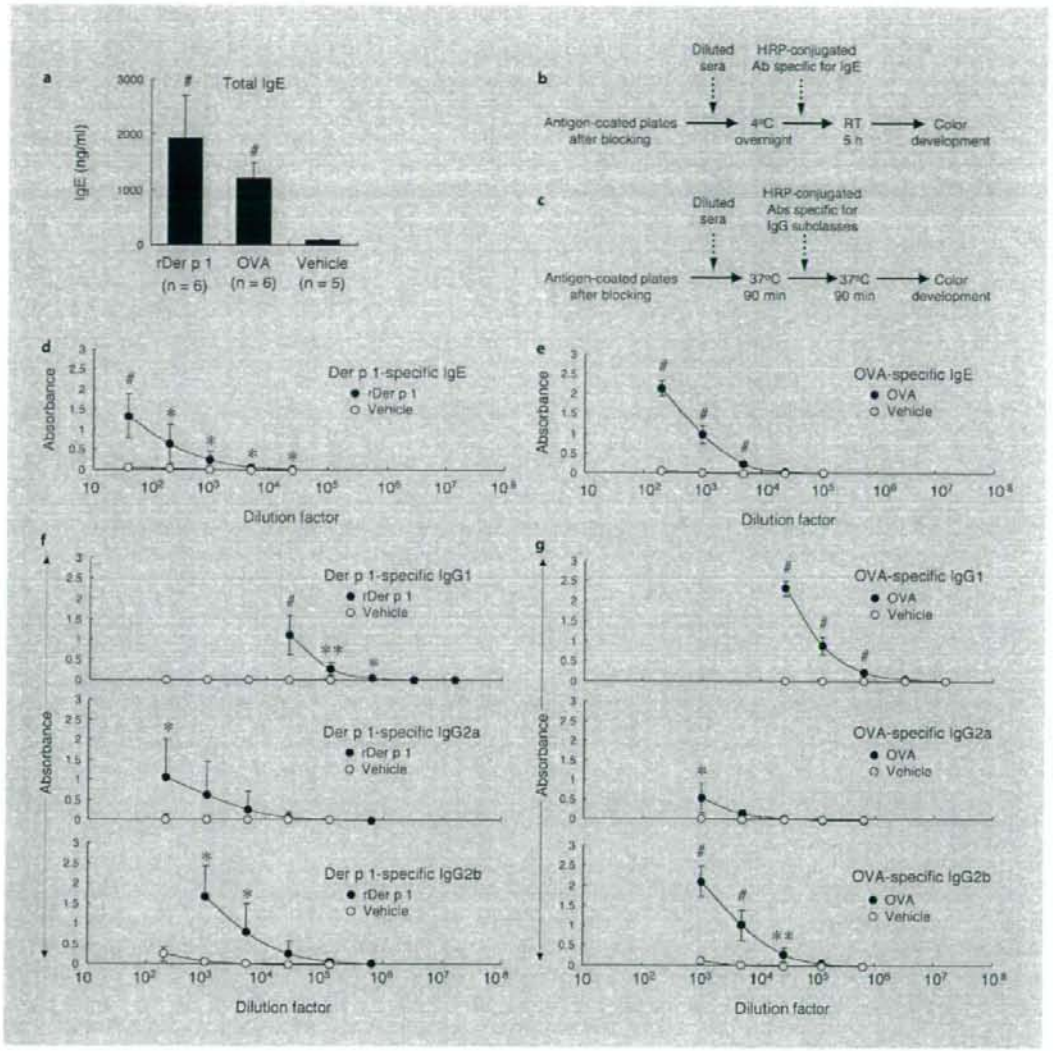
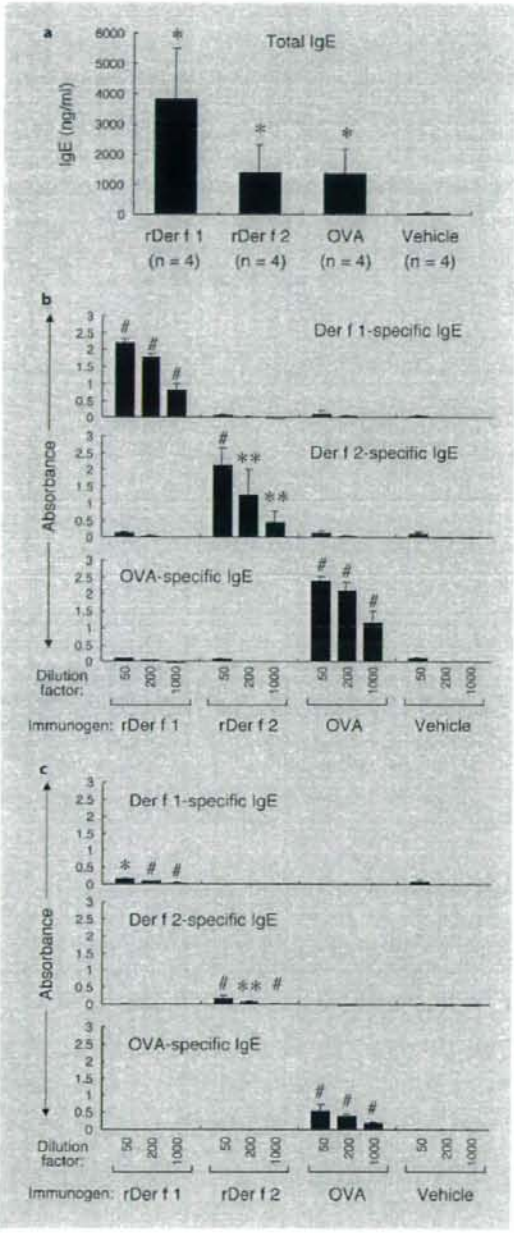


Fig. 1. In-well pretreatment of sera from immunized mice with protein G-coupled sepharose beads enhanced the signals for allergen-specific IgE. Activated rDer f 1 (Der f 1-N53Q, 0.5 μg/head), rDer f 2 (0.5 μg/head), OVA (2.5 μg/head) or buffer alone was mixed with alum and injected into CBA/J mice. **a** Total IgE. **b** Procedure for the pretreatment of sera (1/10 dilution) and detection of allergen-specific IgE. **c** Signals detected for Der f 1-, Der f 2- or OVA-specific IgE. Data shown represent the means ± SD of 7 (rDer f 1 and rDer f 2), 6 (OVA) or 5 mice (vehicle). # $p < 0.001$ vs. vehicle control by unpaired t test (two-tailed). RT = Room temperature.



Fg. 2. Use of the immunoreaction enhancer solutions strengthened the signals for allergen-specific IgE without the removal of IgG. Activated rDer p 1 (Der p 1-N52Q, 2.5 μg/head), OVA (2.5 μg/head) or buffer alone was mixed with alum and injected into CBA/J mice. Enhancer solutions (Can Get Signal; Toyobo) were used for the dilution of the sera and secondary Abs. **a** Total IgE. Procedures for the detection of allergen-specific IgE (**b**) and IgGs (**c**). Signals detected for IgE specific to Der p 1 (**d**) and OVA (**e**) in serially diluted sera. Signals detected for IgG subclasses specific to Der p 1 (**f**) and OVA (**g**). Dilution factor: factors for serum dilution. Data shown represent the means ± SD of 6 (rDer p 1 and OVA) or 5 mice (vehicle). * $p < 0.05$, ** $p < 0.01$ and # $p < 0.001$ vs. vehicle control by unpaired t test (two-tailed). RT = Room temperature.



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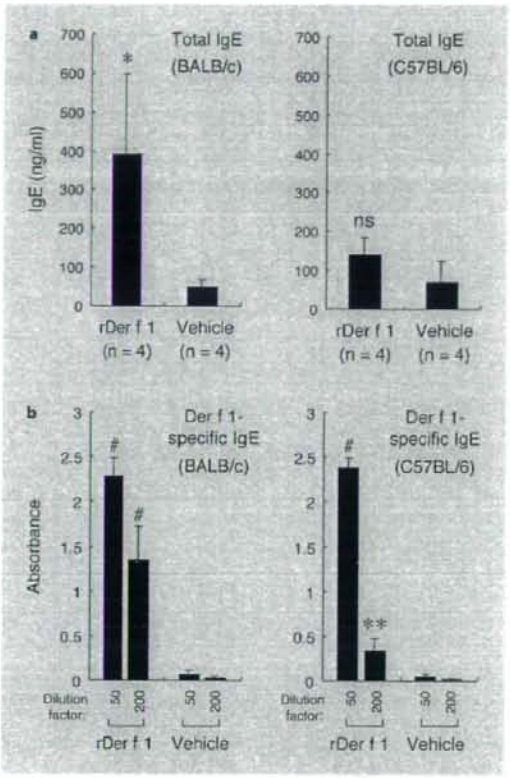

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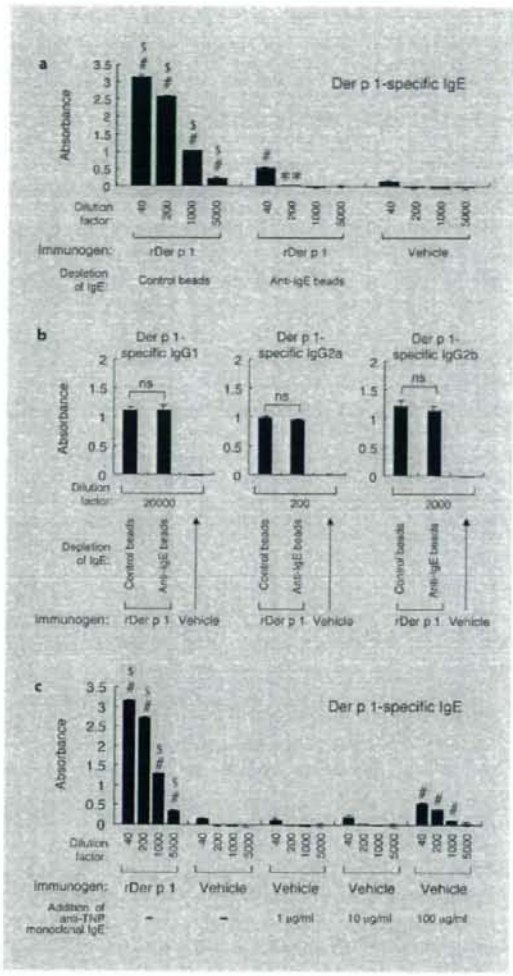
Fig. 3. ELISA using the immunoreaction enhancer solutions exhibited specificity to immunized allergens and high sensitivity for detection. Activated rDer f 1 (Der f 1-N53Q, 0.5 μg/head), rDer f 2 (0.5 μg/head), OVA (1 μg/head) or buffer alone was mixed with alum and injected into CBA/J mice. **a** Total IgE. IgE specific to Der f 1, Der f 2 and OVA detected using enhancer solutions (Can Get Signal; Toyobo) (**b**) or PBST containing a blocking reagent (**c**) for the dilution of the sera and secondary Ab by the procedure shown in figure 2b. Dilution factor: factors for serum dilution. Data shown represent the means ± SD of 4 mice. * $p < 0.05$, ** $p < 0.01$ and # $p < 0.001$ vs. vehicle control by unpaired t test (two-tailed) (**a**) and by the Tukey post hoc test followed by one-way ANOVA (**b**, **c**).

Fig. 4. ELISA using the immunoreaction enhancer solutions effectively detected allergen-specific IgE in other strains of mice. Activated rDer f 1 (Der f 1-N53Q, 10 μg/head) or buffer alone was mixed with alum and injected into BALB/c and C57BL/6 mice. **a** Total IgE. **b** Signals for Der f 1-specific IgE detected by the procedure shown in figure 2b using enhancer solutions (Can Get Signal; Toyobo) for the dilution of the sera and secondary Ab. Dilution factor: factors for serum dilution. Data shown represent the means ± SD of 4 mice. * $p < 0.05$, ** $p < 0.01$ and # $p < 0.001$ vs. vehicle control by unpaired t test (two-tailed). n.s. = Not significantly different ($p > 0.05$).



Fg. 5. Confirmation of isotype specificity to IgE and evaluation of nonspecific binding of IgE to the plate. Activated rDer p 1 (Der p 1-N52Q, 2.5 µg/head) or buffer alone was mixed with alum and injected into CBA/J mice. Two months after the last intraperitoneal injection, the mice were challenged intranasally twice with rDer p 1 (10 µg/head) or buffer alone. Enhancer solutions (Can Get Signal; Toyobo) were used for the dilution of the sera and secondary Abs. Signals for Der p 1-specific Abs detected by the procedures shown in figure 2b for IgE (**a**, **c**) and figure 2c for IgGs (**b**). rDer p 1-immunized pooled serum was treated with anti-IgE beads or control beads. **c** Pooled serum of the vehicle was added to 1, 10 or 100 µg of monoclonal IgE specific to TNP (purified mouse IgE isotype control, clone IgE-3; BD Biosciences)/ml of serum. Dilution factor: factors for serum dilution. Data shown represent the means ± SD for 3 wells of each of the pooled sera. ** $p < 0.01$ and # $p < 0.001$ vs. vehicle (**a**, **c**), $ $p < 0.001$ (control beads) vs. anti-IgE beads (**a**) and $ $p < 0.001$ (rDer p 1) vs. vehicle supplemented with 100 µg/ml of IgE (**c**) by the Tukey post hoc test followed by one-way ANOVA. n.s. = Not significantly different ($p > 0.05$) by unpaired t test (two-tailed) (**b**).

Addition of monoclonal IgE, which is not specific for Der p 1, to the pooled serum of the vehicle control did not significantly increase the signals at 1 and 10 µg of IgE/ml of serum compared with the vehicle control without additional IgE (fig. 5c). Although the signals at 100 µg of IgE/ml of serum were stronger than for the vehicle control, the titer was much lower (approximately 1/25) than with the rDer p 1-immunized serum, which contained a total of 3.5 µg/ml of IgE.

Discussion

A number of methods have been employed to estimate concentrations of allergen-specific IgE in immunized mice, including passive cutaneous anaphylaxis, skin testing [21], degranulation assay using a rat basophilic leukemia cell line [22], immunoblotting, and ELISA using plastic microtiter plates. ELISA for the detection of allergen-specific IgE using allergen-coated plates has the advantage of high throughput, reproducibility of results and simple handling. However, low signal intensity for allergen-specific murine IgE has been a problem, although the total concentration of IgE in serum can be measured by sandwich ELISA (fig. 1a, 2a, 3a, 4a). In the present study, we demonstrated that the in-well pretreatment of murine sera with protein G-coupled sepharose beads (fig. 1c) or the use of commercially available enhancer solutions (fig. 2d, e, 3b, 4b) enhances the signals for the binding of allergen-specific IgE to allergen-coated plates. As the in-well pretreatment of sera with protein G beads needs only a small volume of serum (1/10 dilution, 50 µl of diluted and pretreated serum/well) (fig. 1b) and achieves the simultaneous treatment of many sera, there are advantages over a method using a packed protein G column for the removal of IgG [3]. In the latter system, by simply using enhancer solutions for the dilution of the sera and secondary Ab, a more extensive serum dilution (1/50, 1/200 or less dilution; 50 µl of diluted serum/well) was possible for the detection of the signal and the removal of IgG was not necessary, although the incubation

was prolonged (fig. 2b). The former and latter systems take 2 and 3 days, respectively, from the coating of plates with allergens to measurements of absorbance. Although both methods are effective, the latter has remarkable advantages over conventional methods in terms of signal intensity and handling.

Although the total concentration of IgE on immunization with rDer p 1, rDer f 1 or rDer f 2 was higher than or similar to that on immunization with OVA (fig. 1a, 2a, 3a), the signal for IgE specific to Der p 1, Der f 1 or Der f 2 was weaker than that for OVA-specific IgE (fig. 1c, 2d, e, 3b, c). Although one possible explanation for the results is that the efficiency of the immobilization of Der p 1-WT, Der f 1-WT and rDer f 2 could be lower than that of OVA, other explanations could also be envisaged, such as the fact that the ratio of total to specific IgE could be influenced by the nature of the administered protein. Very recently, we have reported on the commitment of proteolytic activity of rDer p 1 to sensitization towards IgE and IgG responses [23]. Both methods, in-well pretreatment of the murine sera with protein G beads and the use of enhancer solutions, are considered applicable to various allergens.

Pretreatment of the sera with protein G beads enhances signals for allergen-specific IgE because of the removal of allergen-specific IgG, which competitively inhibits the binding of allergen-specific IgE to the allergen immobilized on the plates. In our preliminary experiments, more than 95% of the allergen-specific IgG1 and approximately 20% of the total IgE were removed by the pretreatment (unpubl. data). However, in the case of the mice used for the experiment in figure 5, some individual sera showed strong signals for Der p 1-specific IgE even without the treatment with protein G beads and the signals were equivalent in intensity to those for sera with the treatment, suggesting that a different administration protocol might influence the affinity and amount of allergen-specific IgE and the ratio of allergen-specific IgE versus allergen-specific IgG, which are considered to affect the intensity of the signal for allergen-specific IgE.

The enhancer solutions remarkably strengthened the signals for allergen-specific IgE in spite of the presence of abundant allergen-specific IgG (fig. 2f, g). The composition of the enhancer solutions and the mechanism for the signal enhancement have not been revealed by the manufacturer. Ionic strength or some additives might be designed to stabilize the molecular interaction between the antigen and Ab and between the Ab and secondary Ab to decrease the dissociation rate in the enhancer solutions. In our experience, the use of the second of the enhancer solution 2 seems critical to the enhancement of signals for allergen-specific IgE, although both solution 1 and solution 2 contribute to the enhancement (unpubl. data). The IgE in mice immunized with an allergen did not bind to allergens with which the mice were not immunized, indicating that the binding of IgE to the plates is allergen-specific and nonspecific binding is negligible (fig. 3b, c). Results using serum depleted of (fig. 5a, b) or supplemented with IgE (fig. 5c) also support the isotype specificity of IgE and indicate that the nonspecific binding of IgE to the plate is negligible below a total IgE concentration of 10 μg/ml and that titration will be useful for the appropriate evaluation of allergen-specific IgE even at higher concentrations such as 100 μg/ml. The ELISA system using the enhancer solutions is applicable to sera from 3 mouse strains with differences in genetic background, the strength of the IgE response and the optimal dose of antigen to induce a maximum IgE response. Upon rDer f 1 immunization, CBA/J mice (H-2^k) showed a higher IgE response even at a relatively low dose (fig. 1–3), and BALB/c (H-2^d) and C57BL/6 mice (H-2^b) showed a lower response (fig. 4). Furthermore, even though no significant increase in the total concentration of IgE, which includes nonspecific IgE and allergen-specific IgE, was observed in C57BL/6 mice immunized with rDer f 1 (fig. 4a, C57BL/6), a significant increase in the level of Der f 1-specific IgE was detected (fig. 4b, C57BL/6), implying that the signal for allergen-specific IgE measured by the ELISA system is highly sensitive and more reliable as an indicator of whether the murine model tested is suitable for the study of allergies than total IgE.

In summary, we evaluated the effects of the in-well pretreatment of sera with protein G-coupled sepharose beads and the use of immunoreaction enhancer solutions, which have recently become commercially available, on the enhancement of the signals for the binding of allergen-specific IgE to plates coated with recombinant major house dust mite allergens or OVA. Although we demonstrated that both methods are effective and did not compare them under the same conditions, the use of enhancer solutions is more convenient because it allows the detection of the signals at more extensive dilutions of sera and needs no protein G treatment. An ELISA simply using the enhancer solutions, which obtains remarkably strengthened signals for allergen-specific IgE, would be useful for the study of allergies with murine models. In addition, this paper showed that recombinant forms of the major mite allergens Der p 1, Der f 1 [12–17, 23] and Der f 2 [18–20, 24] can elicit a significant allergen-specific IgE response in mice.

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IL-21–induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses

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Epidemiological studies have suggested that the recent increase in the incidence and severity of immunoglobulin (Ig)E-mediated allergic disorders is inversely correlated with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) vaccination; however, the underlying mechanisms remain uncertain. Here, we demonstrate that natural killer T (NKT) cells in mice and humans play a crucial role in the BCG-induced suppression of IgE responses. BCG-activated murine Vα14 NKT cells, but not conventional CD4 T cells, selectively express high levels of interleukin (IL)-21, which preferentially induces apoptosis in Bε cells. Signaling from the IL-21 receptor increases the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor, resulting in Bε cell apoptosis. Similarly, BCG vaccination induces IL-21 expression by human peripheral blood mononuclear cells (PBMCs) in a partially NKT cell–dependent fashion. BCG-activated PBMCs significantly reduce IgE production by human B cells. These findings provide new insight into the therapeutic effect of BCG in allergic diseases.

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Abbreviations used: α-GalCer, α-galactosylceramide; BCG, *Mycobacterium bovis* bacillus Calmette Guerin; BM-DC, BM-derived DC; Bmf, Bcl-2–modifying factor; γc, common γ-chain; IRAK, IL-1R–associated kinase; MNC, mononuclear cell; MyD88, myeloid differentiation factor 88; PGN, peptidoglycan; TLR, Toll-like receptor.

The prevalence of IgE-mediated allergic diseases such as asthma, hay fever, and atopic dermatitis has increased dramatically over the past two decades, especially in industrialized countries (1). For example, the incidence of asthma has nearly doubled since 1980 in the United States as well as in Japan (1, 2). However, the precise mechanisms underlying the increased incidence of allergic diseases are not fully understood. One possible explanation has been termed "the hygiene hypothesis," which proposes that improved hygiene combined with the excessive use of antibiotics in industrial countries has markedly reduced the incidence of infections, particularly in children. This lack of early exposure to infectious agents is associated with accelerated IgE production and an increased incidence of allergic disorders (1–3). Epidemiological studies support this hypothesis (4–6), and bacterial and viral products have been proposed as therapeutic strategies to suppress the development of allergic responses. For example, vaccination with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) has been reported to suppress IgE production and inhibit the development of allergic diseases in mouse models (7–9) and in humans (10). Furthermore, injection of CpG oligodeoxynucleotides, bacterial DNA surrogates recognized by Toll-like receptor (TLR)9, reduces serum IgE levels in mice (11).

It has been widely accepted that IgE production is totally dependent on Th2 cells, whose functions are reciprocally inhibited by Th1 cells. Mechanistically, therefore, the hygiene hypothesis is based on an imbalance in

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