Ovalbumin immunization

Three weeks after starting the administration of either control or PKGM diet, the mice (female) were immunized intraperitoneally with ovalbumin (OVA) (Sigma, St Louis, MO, USA, 2 μg/mouse) with an alum adjuvant (LSL, Tokyo, Japan). Two and four weeks after the first immunization, the mice were given boosters using the same doses of the antigen. After the first challenge, and 1 week after the last challenge, plasma was obtained by as described above.

Plasma concentrations of total IgE antibody were determined by ELISA as previously described [14]. For the measurement of OVA-specific plasma IgE, microtitre plates were coated with OVA (100 μg/mL), incubated with plasma, and then with a biotinylated rat anti-mouse IgE (BD Bioscience, San Jose, CA, USA, 1 : 250 dilution). Finally, AttoPhos Substrate (Boehringer, Mannheim GmbH, Mannheim, Germany) was added after adding streptavidin-labelled alkaline phosphatase (BD Bioscience, 1 : 1000 dilution), and the fluorescence intensity was monitored by CytoFluor (Excitation: 485/20, Emission: 590/35, Applied Biosystems, Foster City, CA, USA). Serial dilutions of mouse IgE standard (BD Bioscience) were used for a standard curve and were measured in the same way as plasma samples except for using the plate coated with anti-mouse IgE (BD Bioscience). One unit was regarded as equivalent to 1 ng/mL mouse IgE from the standard curve.

Measurement of gene expression levels by real-time reverse transcriptase-polymerase chain reaction

Spleens from control-fed and PKGM-fed mice injected with either PBS or PAM extract six times were isolated one week after the last injection. The levels of ε germline transcription, IL-4, IFN-γ, GATA-3 and T bet expression in the spleen were evaluated by quantitative real-time RT-PCR (TaqMan assay) [20]. The assays were performed with ABI PRISM 7700 sequence detector (Applied Biosystems). Total RNA from each spleen was isolated with RNeasy Mini kit (QIAGEN K.K., Tokyo, Japan). Total RNA was subjected to the first-strand complementary DNA (cDNA) synthesis by a Superscript II first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Primers and probe for ε germline transcription were synthesized by QIAGEN Custom Oligo (Tokyo, Japan), as detailed in a previous report [21]. Primers and TaqMan minor groove binder probes for IL-4, IFN-γ, GATA-3 and T bet genes were obtained from Applied Biosystems (TaqMan Gene Expression Assays). The measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels, as an internal standard for calibration, was performed with TaqMan Rodent GAPDH Control Reagents (Applied Biosystems). The gene expressions were quantified by relating the PCR threshold cycle obtained from tissue samples to amplicon-specific standard curves. Amplification was performed for one cycle of a sequential incubation at 50 °C for 2 min and 95 °C for 10 min, and subsequent 60 cycles of consecutive incubations were performed at 95 °C for 15 s and 60 °C for 1 min.

Statistics

Data were presented as means ± SEM. The significance of differences between treated groups, and that among different time points in each group were determined by two-way ANOVA, and one-way ANOVA, respectively. Post hoc analyses were performed by Bonferroni's post-test. The significance of differences in gene expression was determined by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

The effect of pulverized Konjac glucomannan on the levels of plasma immunoglobulin in BALB/c mice

To investigate the role of PKGM in the modulation of humoral immune responses *in vivo*, we examined plasma concentrations of Igs in BALB/c mice which were fed either mice diet containing 5% PKGM (w/w) or an isocaloric diet, *ad libitum*, and injected with PAM extract or PBS every 2 weeks.

After 14-weeks feeding of either control or PKGM diet, there was no difference in the appearance and body weight among the groups (30.2 ± 0.5 g for PBS-injected mice fed with control diet, 29.5 ± 0.4 g for PBS-injected mice fed with PKGM diet, 30.7 ± 0.3 g for PAM extract-injected mice fed with control diet, 30.0 ± 0.3 g for PAM-extract-injected mice fed with KGM diet). In accordance with our previous report [17], continual injection of PAM extract induced the increase of plasma IgG and IgE levels during the period of the experiment in mice fed with control diet. The levels of plasma IgG and IgE in mice fed with PKGM were significantly lower than those in mice fed with control diet: IgG at 5 and 11 weeks (Fig. 1b) and IgE at 9 and 11 weeks (Fig. 1c). However, they were still significantly higher than those in mice injected with PBS. Neither PAM extract injection nor PKGM diet affected the plasma levels of IgM and IgA (Figs 1a and d). To further study the effect of PKGM on IgG, the levels of IgG subclass (IgG1, IgG2a, IgG2b, IgG3) in each group were measured by IgG subclass-specific ELISAs. The increase of plasma IgG1, which was enhanced to the greatest extent by the injections with PAM extract in accordance with our previous report, was totally abolished in mice fed with PKGM diet (Fig. 2a). The levels of IgG2b in mice injected with PAM extract were also suppressed to some extent in PKGM-fed mice injected with PAM extract at 11 weeks (Fig. 2c). Plasma IgG3 levels in PKGM-fed mice were significantly higher than in control-fed mice (Fig. 2d). Neither PAM extract nor PKGM diet affected the levels of plasma IgG2a (Fig. 2b).

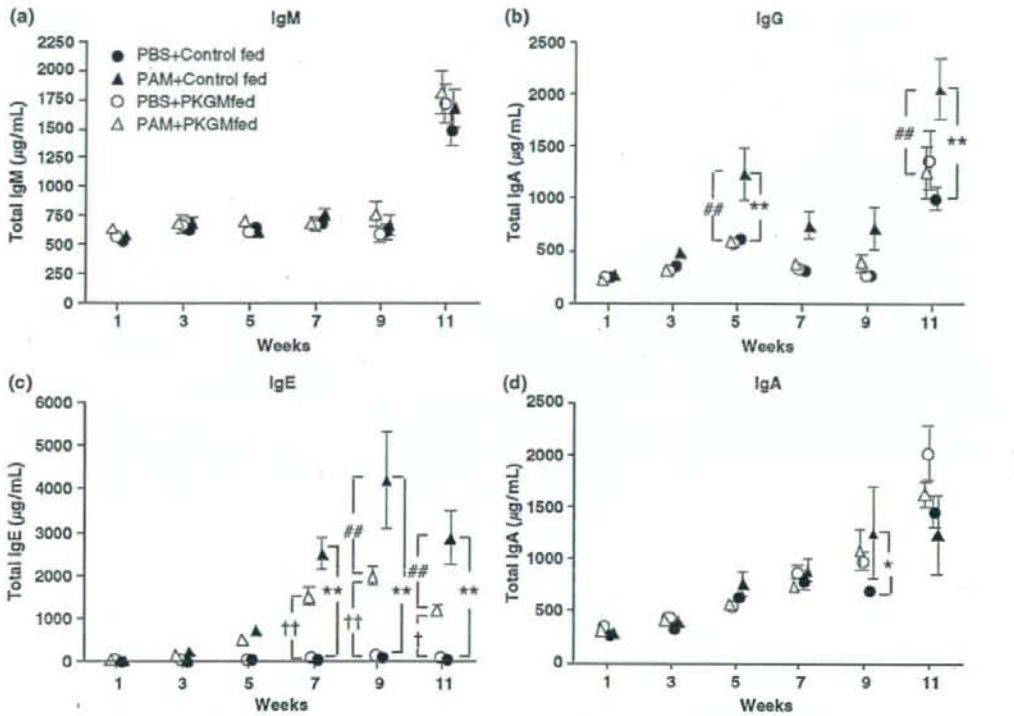

Fig. 1. Effect of pulverized Konjac glucomannan (PKGM) on the levels of plasma immunoglobulins (Igs) in BALB/c mice injected with PAM-extract. BALB/c mice, fed with control diet (closed circle: $n = 9$; closed triangle: $n = 8$) or PKGM-containing diet (5%, w/w; open circle: $n = 10$; open triangle: $n = 8$) were injected with either PAM extractor PBS every 2 weeks. Plasma Ig levels were measured by ELISA every one week after each injection. Injections of PAM extract increased the plasma levels of IgE (c), IgG (b) and IgA (d), but not IgM (a), in the mice fed with control diet (closed circle vs. closed triangle). However, plasma concentrations of IgE (c) and IgG (b) in PKGM-fed mice (open triangle) were significantly lower than those in control-fed mice (closed triangle) in spite of the injection of PAM extract. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ PBS+control-fed group vs. PAM+control-fed group. $^{\#\#}P < 0.01$ PAM+control-fed group vs. PAM+PKGM-fed group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ PBS+PKGM-fed group vs. PAM+PKGM-fed group.

To confirm the effect of PKGM on antigen-specific IgE, we examined the plasma concentrations of total and OVA-specific IgE in BALB/c mice that were sensitized with OVA and fed with either PKGM or control diet. Both total IgE and OVA-specific IgE in PKGM-fed mice were significantly lower than those in control-fed mice (Table 1), indicating that PKGM inhibited antigen-induced immunoresponses.

The effect of pulverized Konjac glucomannan on immunoglobulin E class switching

To study the effect of PKGM on IgE class switching, the amount of ε germline transcript in spleen of each group of mice at 11 weeks after the start of injections with either PAM extract or PBS were determined by quantitative real-time RT-PCR. No obvious difference was observed in the size of the spleen among groups (data not shown).

The level of ε germline transcript in mice injected with PAM extract was attenuated in mice fed with PKGM diet (Fig. 3). Administration of PKGM also inhibited ε germline transcription in mice injected with PBS. There was no difference of ε germline expression between mice injected with PAM extract and those with PBS, when mice were fed with PKGM diet. This result indicated that PKGM prevented not only the increase of plasma IgE, but also the class switching in B cells to produce IgE.

The effect of pulverized Konjac glucomannan on the expression of mRNA for interleukin-4, interferon-γ, GATA-3 and T bet in the spleen of BALB/c mice injected with PAM-extract

The expression levels of mRNA for IL-4, IFN-γ, GATA-3 and T bet in the spleen of mice injected with PAM extract at 11 weeks were determined by quantitative real-time RT-PCR. As expected, both the levels of mRNA for IL-4 and GATA-3 in mice injected with PAM extract were significantly suppressed when mice were fed with PKGM

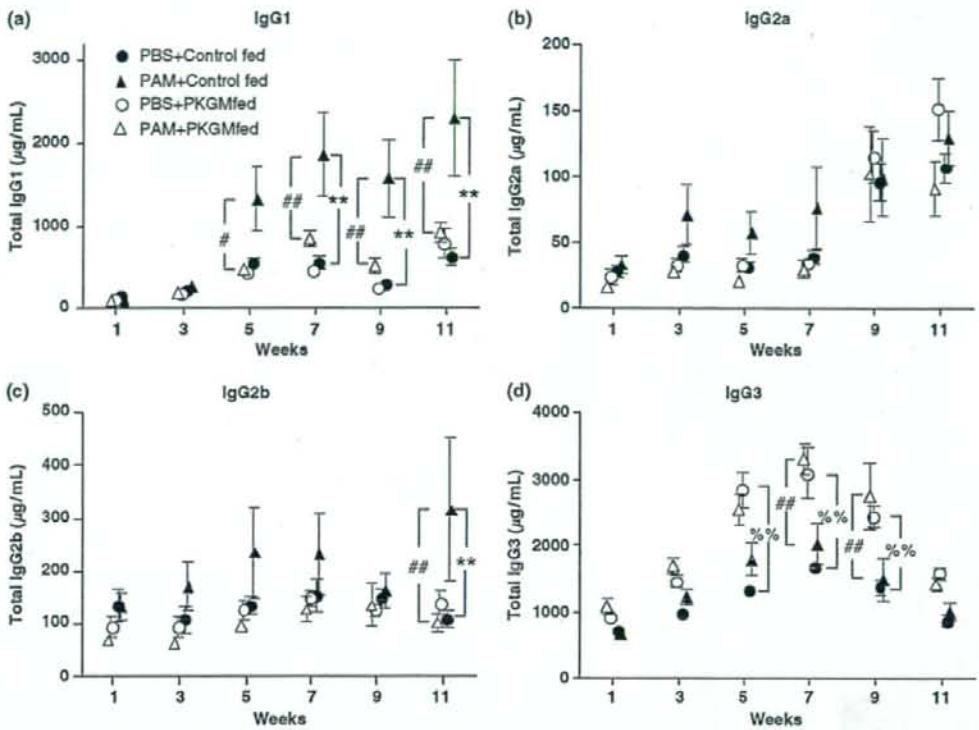

Fig. 2. Effect of pulverized Konjac glucomannan (PKGM) on the levels of plasma immunoglobulin (Ig)G subclasses in BALB/c mice injected with PAM-extract. The levels of plasma IgG subclasses of mice described in Fig. 1 were measured by ELISA, and are shown with symbols as described in Fig. 1. Among the IgG subclasses, the increase of IgG1 concentration was the most dramatically inhibited and the difference between that of control-fed mice and that of PKGM-fed mice became significant at 5 weeks and then after (a). The increases of plasma IgG2b in control-fed mice injected with PAM extract (closed triangle) were significantly inhibited in PKGM-fed mice (open triangle) at 11 weeks (C). The levels of IgG3 in mice given by PKGM administration were higher than those by control chaw administration either when injected with PAM extractor phosphate-buffered saline (PBS) (d). No significant effects of PKGM feeding on the plasma IgG2a were observed (b). $^{**}P < 0.01$ PBS+control-fed group vs. PAM+control-fed group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ PAM+control-fed group vs. PAM+PKGM-fed group. $^{\%\%}P < 0.01$ PBS+control-fed group vs. PBS+PKGM-fed group.

diet (Figs 4a and c). However, the expression of mRNA for IFN-γ either in mice injected with PBS or those with PAM extract was also suppressed when the mice were fed with PKGM diet (Fig. 4b). Moreover, the expression of mRNA for T bet in mice fed with PKGM diet was lower than that in mice fed with control diet, especially in the group injected with PBS (Fig. 4d). There was no difference of the levels of the four genes between mice injected with PAM extract and those with PBS, when mice were fed with PKGM diet. These results suggested that PKGM suppressed not only Th2 response but also Th1 response of the lymphocytes.

Discussion

In this study, we demonstrated that the administration of PKGM diet reduced the increase of plasma IgE, IgG1 and IgG2b induced by the injection of the soluble fraction of syngeneic keratinocytes (PAM-extract) in BALB/c mice. Neither PAM extract nor PKGM diet affected the level of plasma IgM. Moreover, PKGM suppressed not only the plasma levels of total IgE, but also that of OVA-specific IgE in OVA-sensitized mice. The amount of ε germline transcript in the spleen of PKGM-fed mice was significantly lower than that in the spleen of control-diet-fed mice at 11 weeks after the injections. The increases of splenic gene expression for IL-4 and GATA-3 in mice induced by the injection with PAM extract were attenuated when mice were fed with PKGM diet. Interestingly, the expression of mRNA for IFN-γ in mice fed with PKGM diet was also suppressed, regardless of the injection with PAM extract or that with PBS.

The induction of isotype switching to a particular heavy chain in B cell correlates with the transcriptional

Table 1. Effect of PKGM on the plasma level of OVA-specific IgE in mice sensitized with OVA

Weeks	PBS+control fed	PBS+PKGM fed	OVA+control fed	OVA+PKGM fed
Total IgE (ng/mL)				
0	135 ± 27	75.4 ± 8.7	89.7 ± 11	80.1 ± 8.0
5	168 ± 66	108 ± 28	2089 ± 349**	725 ± 206††##
OVA-specific IgE (U)				
0	36.3 ± 0.50	38.8 ± 1.1	36.2 ± 0.41	36.1 ± 0.54
5	136 ± 0.61	138 ± 0.61	2249 ± 307**	917 ± 38††##

Plasma levels of OVA-specific and total IgE in mice injected with OVA or PBS were measured by ELISA. Values are presented as means ± SEM, $n = 6$ per group.

**$P < 0.01$ PBS+control-fed group vs OVA+control-fed group.##$P < 0.01$ OVA+control-fed group vs OVA+PKGM-fed group.††$P < 0.01$ PBS+PKGM-fed group vs. OVA+PKGM-fed group.

PKGM, pulverized Konjac glucomannan; OVA, ovalbumin; IgE, immunoglobulin E; PBS, phosphate-buffered saline.

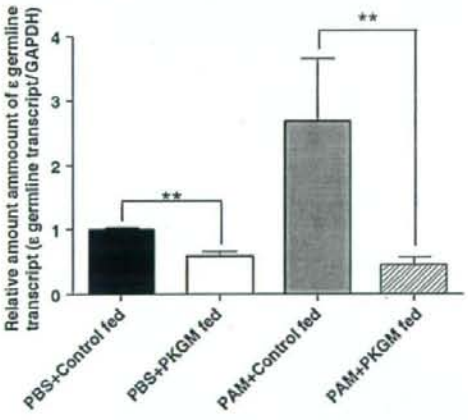

Fig. 3. Effect of pulverized Konjac glucomannan (PKGM) on the level of ε germline transcription in spleen of BALB/c mice injected with PAM-extract. Control- or PKGM-fed mice were injected with PAM extractor phosphate-buffered saline (PBS) as described in Fig. 1. Total RNA was isolated from spleens of the mice 1 week after the final injection at 11th weeks. Epsilon germline transcripts were measured with semi-quantitative real-time RT-PCR. The level of ε germline was normalized by GAPDH, and expressed as a ratio to that in control-fed mice injected with PBS (relative value, 1.0). The level of ε germline transcript in control-fed mice injected with PAM extract (shaded bar; $n = 7$) was dramatically suppressed in mice fed with PKGM (hatched bar; $n = 7$). The level of ε germline transcript in control-fed mice injected with PBS (closed bar; $n = 7$) was also suppressed with PKGM (open bar; $n = 9$). Data are means ± SEM. **$P < 0.01$ by Student's *t*-test.

activation of the corresponding gene in germline configuration [22]. In the case of IgE, ε germline transcription is essential for IgE class switching [23, 24]. In this study, we have demonstrated that the ε germline transcription in the spleen was suppressed in mice fed with PKGM. Hence, the IgE class switching in splenic B cells was inhibited by the administration of PKGM. Although we have not assessed the effect of PKGM on γ1 germline transcription, PKGM likely inhibits the class switching to IgG1 as well, since switching to IgG1 and switching to IgE are potently stimulated by IL-4 [10, 25].

In general, Th2 cells drive B cells to differentiate and produce Igs by secretion of cytokines such as IL-4 and IL-13. In addition, our previous study has shown that the injections of PAM extract consistently increased the levels of serum IgE, IgG1 and IgG2b [17], suggesting the enhancement of Th2 cellular responses. Thus, the results of this study suggested that PKGM suppresses Th2 cellular responses. However, the studies of mRNA expression in the spleen for IFN-γ, IL-4, GATA-3 and T bet suggest rather bi-directional suppression of helper T cells. Recent molecular-biological studies regarding differentiation of helper T cells have revealed that GATA-3 is a potent transactivator of the gene of IL-4 and functions as a Th2 lineage determinant [26, 27]. On the other hand, the expression of T bet mRNA correlates with the production of IFN-γ by Th1 cells [12]. The present results demonstrated that oral administration of PKGM attenuated the splenic gene expressions not only for IL-4 and GATA-3, but also for IFN-γ in mice injected with PAM extract. Moreover, the expressions of both IFN-γ and T bet mRNA were suppressed in mice injected with PBS and fed with PKGM. Onishi et al. [14] have reported the decrease of IFN-γ in plasma of NC/Nga mice fed with PKGM. Taking into account the increase of IFN-γ in chronic stage of atopic dermatitis [28, 29], such bi-directional effects of PKGM may be more beneficial from the view point of the treatment of atopic diseases.

The effects of PKGM are not likely because of a non-specific immunosuppression, because of the following observations. First, PKGM suppressed neither the mRNA expression for IL-4 nor that for GATA-3 in mice injected with PBS. Second, the levels of all plasma Igs in mice fed with PKGM were not different from those in mice fed with control diet, when they were not injected with PAM extract. Third, there was no difference of plasma corticosteroid concentrations between mice fed with control diet and those fed with PKGM (data not shown). Thus, the effect of PKGM is likely a selective modification of helper T cell activities and consequent subsidence of B cell hyper-reactivity rather than a non-specific immunosuppression.

The essential structure in PKGM for immunomodulatory function and its direct target of PKGM is a matter of the discussion. Mice and humans do not have enzymes to digest and metabolize KGM by themselves. However, intestinal bacteria that can degrade KGM were found in humans [30], and may ferment KGM to mono/oligosaccharides in the intestine. Moreover, Onishi et al. [14] have shown that non-pulverized KGM did not inhibit

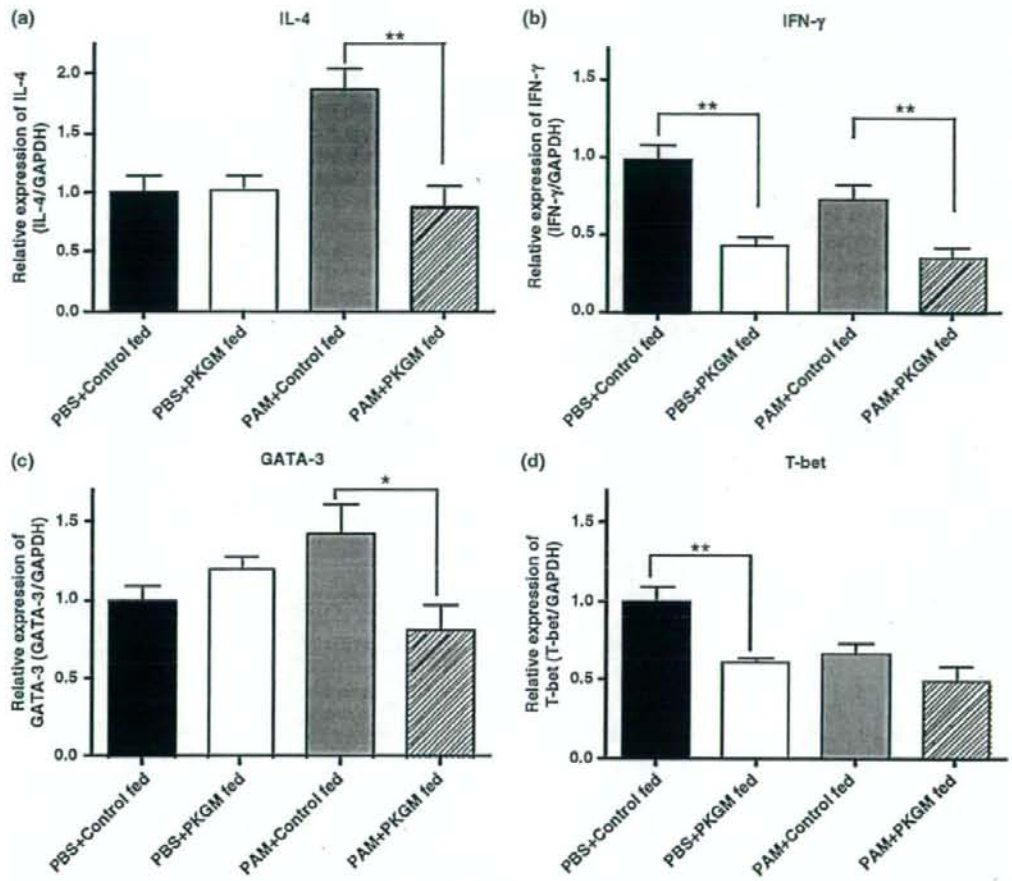

Fig. 4. Effect of pulverized Konjac glucomannan (PKGM) on the levels of mRNA for IL-4, IFN-γ, GATA-3 and T bet in spleen of BALB/c mice injected with PAM-extract. The levels of gene expression were measured as described for Fig. 3 and are shown with bars as described in Fig. 3. The levels of IL-4 (a), IFN-γ (b) and GATA-3 (c) mRNA in control-fed mice injected with PAM extract were significantly suppressed in PKGM-fed mice injected with PAM-extract. The levels of IFN-γ (b) and T bet (d) mRNA in control-fed mice injected with phosphate-buffered saline (PBS) were also significantly suppressed in PKGM-fed mice injected with PBS. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test.

the increase of IgE, suggesting that the pulverization of KGM improved the efficiency of KGM degradation by intestinal bacteria, and enabled KGM to be absorbed by gut mucosae.

The differentiation of naive $CD4^+$ T cells into Th1 and/or Th2 cells is induced by the antigen presentation by antigen-presenting cells (APC), which are essential for the initiation of GATA-3 and T bet gene expression [11, 31]. These processes of differentiation and Th1 and Th2 cell functions are negatively regulated by $CD4^+$ $CD25^+$ regulatory T cells [32]. Therefore, PKGM may influence (APC) or regulatory T cells to inhibit the commitment of splenic lymphocytes to Th1 or Th2. On the other hand, PKGM significantly decreased ε germline transcription without suppressing a IL-4 and GATA-3 expression in control-diet-fed mice injected with PBS, suggesting direct effect of PKGM on B cells. Moreover, IgE production by B cells treated by IL-4 and anti-CD40 antibodies was weakly inhibited in the presence of solubilized KGM, *in vitro* (Suzuki et al., manuscript in preparation). Taken together, PKGM may act on both helper T cells and B cells to reduce the IgE production from B cells.

The level of only IgG3 was increased among those in plasma IgG subtypes by PKGM administration. Recent studies have revealed that IL-21 stimulates both IgG3 production and γ3 germline transcription [33], and prevents antigen-induced IgE production and ε germline transcription [34, 35]. These effects are similar to those of

PKGM observed in this study. However, IL-21 also stimulates the production of IgG1 [33], which was suppressed by PKGM. Likewise, we could not attribute the whole effect of PKGM to any single cytokine, including IL-21. *In vitro* studies for PKGM action on IgE production should elucidate more precise mechanism.

In conclusion, we revealed that oral administration of PKGM suppressed the increase in plasma IgE (both total and antigen specific), IgG1, IgG2b and IgE class switching induced by the injection of syngeneic keratinocyte extracts. PKGM also attenuated the increase of mRNAs for IL-4 and GATA-3 and suppressed the expression of IFN-γ and T bet to some extent. Thus, PKGM may have a profound therapeutic potential against diseases mediated by the increase of IgE and/or the imbalance of helper T cell functions.

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References

1 Leung DY. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol* 1995; 96:302–18.
2 Jones HE, Inouye JC, McGerity JL, Lewis CW. Atopic disease and serum immunoglobulin-E. *Br J Dermatol* 1975; 92:17–25.
3 Casolaro V, Georas SN, Song Z, Ono SJ. Biology and genetics of atopic disease. *Curr Opin Immunol* 1996; 8:796–803.
4 Stone SP, Muller SA, Gleich GJ. IgE levels in atopic dermatitis. *Arch Dermatol* 1973; 108:806–11.
5 Gurevitch AW, Heiner DC, Reisner RM. IgE in atopic dermatitis and other common dermatoses. *Arch Dermatol* 1973; 107:712–5.
6 Clendenning WE, Clack WE, Ogawa M, Ishizaka K. Serum IgE studies in atopic dermatitis. *J Invest Dermatol* 1973; 61:233–6.
7 Werfel S, Massey W, Lichtenstein LM, Bochner BS. Preferential recruitment of activated, memory T lymphocytes into skin chamber fluids during human cutaneous late-phase allergic reactions. *J Allergy Clin Immunol* 1995; 96:57–65.
8 Pandey V, Mihara S, Fensome-Green A, Bolsover S, Cockcroft S. Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosol Ca2+, degranulation, and membrane ruffling in the cultured rat basophilic leukemia-2H3 mast cell line. *J Immunol* 2004; 172:4048–58.
9 Kitaura J, Song J, Tsai M *et al.* Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the FcεRI. *Proc Natl Acad Sci USA* 2003; 100:12911–6.
10 Bacharier LB, Geha RS. Molecular mechanisms of IgE regulation. *J Allergy Clin Immunol* 2000; 105:S547–58.
11 Grogan JL, Locksley RM. T helper cell differentiation: on again, off again. *Curr Opin Immunol* 2002; 14:366–72.
12 Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T bet, directs Th1 lineage commitment. *Cell* 2000; 100:655–69.
13 Kato K, Matsuda K. Studies on the chemical structure of konjac mannan. Part I. Isolation and characterization of oligosaccharides from the partial acid hydrolyzate of the mannan. *Agric Biol Chem* 1969; 33:1446–53.
14 Onishi N, Kawamoto S, Nishimura M *et al.* A new immunomodulatory function of low-viscous konjac glucomannan with a small particle size: its oral intake suppresses spontaneously occurring dermatitis in NC/Nga mice. *Int Arch Allergy Immunol* 2005; 136:258–65.
15 Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 1999; 5:1249–55.
16 Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994; 12:991–1045.
17 Yamamoto T, Kaneko S, Hide M *et al.* Increase in serum IgE levels following injection of syngeneic keratinocyte extracts in BALB/c mice. *Arch Dermatol Res* 2002; 294:117–23.
18 Onishi N, Kawamoto S, Nishimura M *et al.* The ability of konjac-glucomannan to suppress spontaneously occurring dermatitis in NC/Nga mice depends upon the particle size. *Biofactors* 2004; 21:163–6.
19 Yuspa SH, Hawley-Nelson P, Koehler B, Stanley JR. A survey of transformation markers in differentiating epidermal cell lines in culture. *Cancer Res* 1980; 40:4694–703.
20 Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996; 6:986–94.
21 Linehan LA, Warren WD, Thompson PA, Grusby MJ, Berton MT. STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *J Immunol* 1998; 161:302–10.
22 Stavnezer-Nordgren J, Sirlin S. Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J* 1986; 5:95–102.
23 Punnonen J, Aversa G, Cocks BG *et al.* Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993; 90:3730–4.
24 Durham SR, Gould HJ, Thienes CP *et al.* Expression of epsilon germ-line gene transcripts and mRNA for the epsilon heavy chain of IgE in nasal B cells and the effects of topical corticosteroid. *Eur J Immunol* 1997; 27:2899–906.
25 Bergstedt-Lindqvist S, Moon HB, Persson U, Moller G, Heusser C, Severinson E. Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE. *Eur J Immunol* 1988; 18:1073–7.
26 Yoh K, Shibuya K, Morito N *et al.* Transgenic overexpression of GATA-3 in T lymphocytes improves autoimmune glomerulonephritis in mice with a BXSB/MpJ-Yaa genetic background. *J Am Soc Nephrol* 2003; 14:2494–502.
27 Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci USA* 2004; 101:1993–8.
28 Grewe M, Gyufko K, Schopf E, Krutmann J. Lesional expression of interferon-gamma in atopic eczema. *Lancet* 1994; 343:25–6.
29 Grewe M, Bruijnzeel-Koomen CA, Schopf E *et al.* A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 1998; 19:359–61.

30 Matsuura Y. Degradation of konjac glucomannan by enzymes in human feces and formation of short-chain fatty acids by intestinal anaerobic bacteria. *J Nutr Sci Vitaminol (Tokyo)* 1998; 44:423–36.

31 Moser M, Murphy KM. Dendritic cell regulation of TH1–TH2 development. *Nat Immunol* 2000; 1:199–205.

32 Xu D, Liu H, Komai-Koma M *et al.* CD4+CD25+ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice. *J Immunol* 2003; 170:394–9.

33 Pene J, Gauchat JF, Lecart S *et al.* Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J Immunol* 2004; 172:5154–7.

34 Ozaki K, Spolski R, Feng CG *et al.* A critical role for IL-21 in regulating immunoglobulin production. *Science* 2002; 298: 1630–4.

35 Suto A, Nakajima H, Hirose K *et al.* Interleukin 21 prevents antigen-induced IgE production by inhibiting germ line Cε transcription of IL-4-stimulated B cells. *Blood* 2002; 100: 4565–73.

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Decrease in the Allergenicity of Japanese Cedar Pollen Allergen by Treatment with Positive and Negative Cluster Ions

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Key Words
Allergenicity · Cluster ions · *Cryptomeria japonica* · Discharge plasma · Japanese cedar pollen

Abstract
Background: Japanese cedar pollinosis is a severe allergic disease in Japan. The most effective means of decreasing allergic inflammation reactions is still avoidance of the aeroallergen. Recently, a novel air purification system using positively and negatively charged cluster ions was developed to create comfortable living environments. We aimed to assess the ability of existing technology to lower allergenicity of Japanese cedar pollen. ***Methods:*** A Japanese cedar pollen extract was nebulized from the top of a cylindrical container with 2 or 4 ion-generating devices. The extract in a mist was passed through the space filled with or without plasma cluster ions for 90 s, and the ion-treated or nontreated extract was then collected in a Petri dish at the bottom of the container. ***Results:*** The ion-exposed extract was significantly diminished in its reactivities to anti-Cry j 1 or anti-Cry j 2 antiserum and to human allergic sera IgE on ELISA. SDS-PAGE analysis revealed that ion exposure induced protein degradation in the pollen extract. Similarly, the ion treatment impaired about 80% of the binding to pooled sera IgE from patients allergic to Japanese cedar pollen on ELISA inhibition. Furthermore, intracutaneous and conjunctival reaction tests showed a remarkable diminution in the allergenicity of the ion-irradiated extract. ***Conclusion:*** Ion irradiation resulted in a remarkable decrease in in vitro and in vivo allergenicities of atomized Japanese cedar pollen extracts.

Introduction

In proportion to increases in air pollution and allergen exposure, morbidity from allergic diseases such as rhinitis, dermatitis, and asthma is rising [1–4]. Especially Japanese cedar *(Cryptomeria japonica)* pollinosis has developed into a severe health and social problem, because the number of those allergic to Japanese cedar pollen (JCP) amounts to about 14% of Japan's total population [5].

The recognition that a pollinosis is provoked only by two major IgE-binding proteins, pectate lyase (Cry j 1) [6–8] and polymethylgalacturonase (Cry j 2) [9–12] has meant that the elucidation as regards other important allergens has been delayed. Recently, an isoflavone reductase-like protein [13] and chitinase [14] were characterized as additional allergens. We also found that 131 JCP

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allergens were recognized in at least 1 of 40 sera IgE samples from patients with Japanese cedar pollinosis on two-dimensional immunoblotting, and that 4–86 protein spots (mean ± SD = 37 ± 22) among them reacted with the respective patient's IgE [15]. Although it is important to examine which allergen molecule plays a pivotal role in the elicitation of sensitization and allergic symptoms on the basis of its physiological properties, efforts to achieve this have become more laborious with the remarkable increase seen in sensitized allergens.

The environmental control of allergens has been recognized as an integral part of the overall management of allergen-sensitized patients [16]. Various trials designed to reduce exposure to indoor allergens appear indispensable to the maintenance of a high quality environment for patients. In most cases, allergen avoidance (e.g., from house dust mites, pets, cockroaches, and pollens) is accomplished by various cleaning measures within the house. Conflicting reports have been published about the effects of chemical expedients, carpet fresheners, acaricides, and protein-denaturing agents [17–21]. Physical means such as high-efficiency particulate arrest (HEPA) filter vacuum cleaners [22–24] and ionizers [25–28] seem to be more suitable for combating airborne allergens, but their clinical benefit is debatable. Accordingly, a combination of valid means directed at different sites seems a desirable approach, while more appropriate ways to effectively decrease the allergenic activities of airborne allergens in our living environment remain to be developed.

In this study, we utilized a novel air purification system emitting plasma cluster ions to significantly reduce the allergenicity of atomized JCP allergens floating in an atmosphere by passing them through a space filled with plasma cluster ions for about 90 s. We anticipate that this air purification system would have a high potential as an effective means to decrease exposure to airborne allergen for patients with a high risk for developing allergic symptoms.

Materials and Methods

JCP and Chemicals

JCP *(C. japonica)* was collected from Mikado Island, Hiroshima, Japan, in the year 2003 and stored at –30°C until use [13, 15]. All chemicals used were of analytical grade unless otherwise indicated.

Patients

C. japonica pollen-allergic sera were obtained from 40 subjects selected on the basis of clinical history, seasonal symptoms and positive values against *C. japonica* pollen-specific IgE (RAST scores: 2–6), and stored at –30°C until use. All experiments were approved by the Institutional Review Board of Takanobashi Central Hospital and were described in detail to all participants before they provided informed consent and were included in the study.

Preparation of a JCP Extract

JCP (80 g) was suspended in 3 liters of 20 mM phosphate-buffered saline (pH 7.4) for 6 h at 4°C, and the suspension centrifuged at 9,300 *g* for 30 min at 4°C. Ammonium sulfate was added to the supernatant up to 80% saturation. The resultant precipitate was dialyzed against distilled water, and the dialysate was adjusted to 200 ng protein/ml and used as the JCP extract.

Structure of an Ion Generation Device and Determination of Ion and Ozone Densities

In this study we used an ion generation device attached to a domestic air cleaner (Sharp, Osaka, Japan) as described by Nishikawa and colleagues [29–31]. The device consisted of a ceramic dielectric plate, a high-voltage applied electrode, and a ground electrode. Plasma cluster ions were generated at atmospheric pressure by discharge plasma on the surface of the ceramic plate. The energy imparted to molecules in the air by discharge plasma was adjusted to about 5 eV. Ion densities were measured in terms of electrical conductivity of air on a double co-cylinder-shaped detector called a Gerdien condenser (83-1011B, Dan Kagaku, Tokyo, Japan). The density of generated ozone was measured either at a distance of 3 cm from the electrode for a duration of 90 min by an ozone monitor (EG-2001F, Ebara Jitsugyo, Tokyo, Japan) or in 600 ml of sampling volume for 6 min by an ozone detection tube (182U, Komyo Rikagaku Kogyo, Tokyo, Japan) immediately after the experiments.

Nontoxic Effects of Plasma Cluster Ions

The safety test was entrusted to the Mitsubishi Chemical Safety Institute (Tokyo, Japan) and was carried out with reference to the OECD Principles of Good Laboratory Practice [C(97)186Final, 1997]. In brief, rats (8-week-old, n = 10 each, male and female) were exposed to positive ions (25,555/cm^3) and negative ions (28,010/cm^3) for 6 h a day during a period of 28 days. No abnormalities or clinical signs occurred in any of the animals. There were no changes suggesting toxic effects of plasma cluster ion exposure on body weight, food consumption, hematology, blood chemistry, qualitative urinalysis, ophthalmoscopy, or organ weight. Furthermore, necropsy and histopathological examinations found no abnormalities in any animal.

Experimental Apparatus and Ion Exposure

An acrylic cylindrical plastic container (14.5 × 52.5 cm) was equipped with 2 or 4 ion-generating devices (fig. 1). When a high AC pulse voltage (width: 25 μs) was applied 120 times per second at 48, 80, or 140 AC input voltages between the electrodes, plasma cluster ions emerged from the surface of the ceramic dielectric plate. The lifetime of the generated plasma cluster ions in air was approximately 3–5 s. The density of generated ozone was measured at <0.005, <0.005, or 0.025 ppm at a distance of 3 cm from the device in proportion to the applied voltage. To exclude the additional effect of accumulated ozone in our closed experimental conditions, active carbon (Kishida Chemical Co., Osaka, Japan) was used. An ascending current of air was blown at a flow rate of

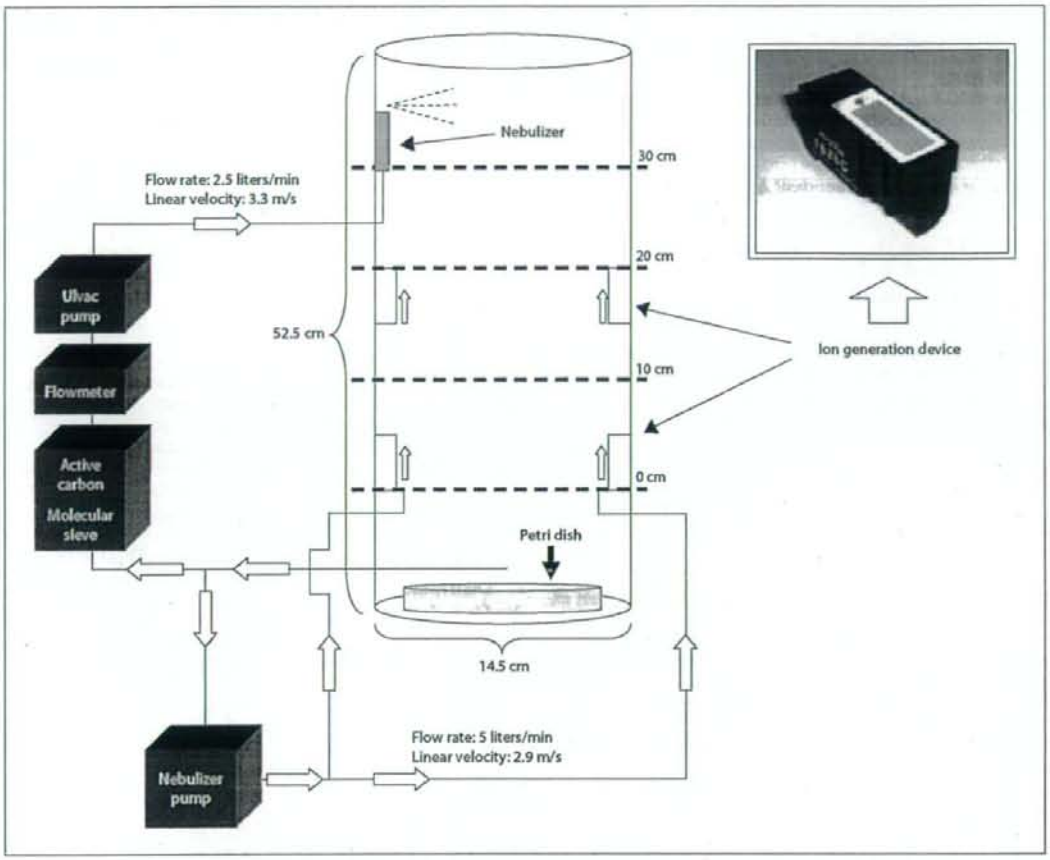

Fig. 1. Exposure system of plasma cluster ions to atomized JCP extract. Two or four ion generation devices (2.15 × 5.7 × 3.1 cm) were attached to a container (Φ14.5 × 52.5 cm), which was filled for 15 min with plasma cluster ions. A JCP extract solution (200 ng/ml) was nebulized from the top of the container using a nebulizer pump and collected in a Petri dish at the bottom. An additional circulation pump was equipped to make an ascending current of air inside the chamber. Air flows are indicated by arrows.

5 liters/min from a nozzle installed on the surface of the ion-generating unit to disperse the plasma cluster ions and was then circulated from the outlet at the bottom of the chamber using a pump (from NE-c16 nebulizer, Omron, Kyoto, Japan). A molecular sieve 4A (Kishida Chemical Co.) and active carbon tubes were inserted in the circulating duct. The molecular sieve and charcoal were replaced every other run. After plasma cluster ions were prefilled for 15 min in the chamber, 8 ml of JCP extract solution (200 ng/ml) was atomized for 1.5 h using a nebulizer pump (Ulvac pump DAP-15, Ulvac KIKO, Kanagawa, Japan). After 1.5 h irradiation, the densities of accumulated ozone measured at the bottom of the chamber by an ozone detector 182U were 0.025 ppm in the chamber when input voltages of 4 ion-generating devices were set at 140 V, but were under the limit of detection in the other cases. A JCP extract in mist passed through a space full of or without plasma cluster ions for 90 s and was collected in a tray (about 1 ml) at the bottom of the container to serve as ion-treated or nontreated JCP extracts, respectively. After the ion-treated and nontreated experiments had been repeated 100 times, no significant difference in total protein contents (the method of Lowry et al. [32]) between the respective recovered and concentrated solutions was recognized. The effect of plasma cluster ions on allergen molecules was

evaluated in terms of changes in the intensities of antigenicity and allergenicity per unit of protein.

SDS-PAGE

Ion-treated and nontreated JCP extracts (50 μg protein each) were run on a 15% SDS gel under reduced conditions. Protein bands were visualized by silver staining.

Enzyme-Linked Immunosorbent Assay and Enzyme-Linked Immunosorbent Assay Inhibition

For determination of Cry j 1 and Cry j 2 contents in a JCP extract with or without ion treatment, 50 μl of the respective JCP extract solutions (100 ng/ml of 100 mM bicarbonate buffer, pH 9.4) was coated on a 96-well microtiter plate (F96 Cert. Maxisorp, Nunc-Immuno plate, Nalge Nunc International, Denmark) and incubated at room temperature for 2 h. After blocking the plate with 3% skim milk and 1% bovine serum albumin in PBST overnight, 1,000-fold diluted polyclonal antibodies against Cry j 1 or Cry j 2 (Seikagaku Kogyou, Tokyo, Japan) were added and incubated for 4 h. The plate was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1,500-fold diluted, New England Biolabs, Beverly, Mass., USA) for 1 h. Next, the plate was stained with 50 μl of a substrate buffer containing 10 mg of ABTS [2,2'-azinobis(3-etylbenzthiazoline-6-sulfonate)] and 10 μl of 30% H_2O_2 in 10 mM citrate buffer at pH 4.2 and measured at 405 nm with a Wallac ARVO™SX plate reader (Perkin Elmer Japan, Yokohama, Japan).

The IgE-binding intensity of JCP extract exposed or not exposed to plasma cluster ions was evaluated by enzyme-linked immunosorbent assay (ELISA) [13]. In brief, 50 μl of a JCP extract solution (100 ng/ml of 100 mM bicarbonate buffer, pH 9.4) was coated on a 96-well microtiter plate and incubated at room temperature for 2 h. After blocking the plate overnight, 50 μl of 10-fold diluted patient sera was added and incubated for 4 h. Then, 50 μl of 1,000-fold diluted biotinylated anti-human IgE (ε-chain-specific, Vector Laboratories, Burlingame, Calif., USA) was added and incubated for 2.5 h. After washing the plate, alkaline phosphatase-conjugated streptavidin (50 μl, Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) was added and incubated for 1.5 h. The washed plate was then stained with an Attophos™ substrate buffer (Roche, Mannheim, Germany) and measured at 485 nm emission and 530 nm excitation using a CytoFluor II microplate reader (PE Biosystems, Norwalk, Conn., USA).

ELISA inhibition was carried out with ion-treated or nontreated JCP extract solutions against pooled sera IgE (n = 5) from patients with Japanese cedar pollinosis [13]. In brief, 50 ng of an intact JCP extract in 50 μl of 100 mM $NaHCO_3$ at pH 9.4 was coated onto a 96-well plate for 2 h. Beforehand, 20-fold diluted pooled sera were reacted with 50 μl of ion-treated or nontreated JCP extract solutions at concentrations of 8 series of 5 times dilutions from a starting concentration of 11 μg/ml overnight at 4°C. The incubation mixture was added to the coated plate. Subsequent procedures were the same as described above.

Intracutaneous Test

Intracutaneous tests were carried out according to a previously reported method [33, 34]. Briefly, 20 μl of ion-treated or nontreated JCP extract solutions (500 ng/ml in 0.9% NaCl) were injected in patients with Japanese cedar pollinosis. Scores of –, ±, +, ++ and +++ were assigned for erythema of <10, 10 to <20, 20 to <30, 30 to <40, and ≥40 mm in mean diameter, respectively, after 15 min.

Conjunctival Reaction Test

Conjunctival reaction tests were carried out in patients who received intracutaneous tests. In accordance with a previously described procedure [33, 35], one lower eyelid of each patient was challenged with 5 μl of a nontreated JCP extract in saline (5 μg/ml) as a control. The other eye of the patient was treated with the same amount of an ion-treated JCP extract. Conjunctiva was compared with the control eye to examine for congestion after 15 min. Scores of –, +, ++ and +++ were assigned for the degree of congestion observed as none, localized, intermediate, and strong, respectively.

Statistical Analysis

Data were analyzed by the difference between means. All experiments were repeated at least 3 times to confirm their reproducibility, and typical results are shown as figures.

Results

Density Distribution of Plasma Cluster Ions

Distribution of ion density was measured for 90 min under a circulating airstream (5 liters/min) in an acrylic cylindrical plastic chamber after two ion generation devices were run for 15 min (fig. 1). When the input voltage of the unit was set at 140 V, the densities of positive ions were detected at 10,070 ± 64, 40,010 ± 70, 80,040 ± 120 and 100,010 ± 2,256 counts/cm^3 (n = 10) at a distance of 25, 20, 15 and 10 cm from the unit, while those of negative ions were at 10,020 ± 87, 49,960 ± 102, 79,980 ± 117 and 100,000 ± 2,049 counts/cm^3, respectively. The ion densities were reduced with decreasing applied voltages (data not shown). To maintain a constant level of plasma cluster ion concentration in the experimental chamber, the number of ion generation devices was increased from 2 to 4 units.

Decrease in in vitro Antigenicities of JCP Extract Treated with Plasma Cluster Ions

First, we examined the change in rabbit IgG-reactive epitopes in Cry j 1 or Cry j 2 molecules induced by exposure of a JCP extract to plasma cluster ions generated from four ion-generating devices. Significant reduction in IgG-binding capacities of the major allergens, Cry j 1 and Cry j 2, was observed in ion-treated JCP extract (paired t test, $p < 0.05$), compared with nontreated JCP extract (fig. 2).

Next, we investigated whether changes in overall human IgE epitopes took place in JCP extract. The treatment of JCP extract with plasma cluster ions significant-

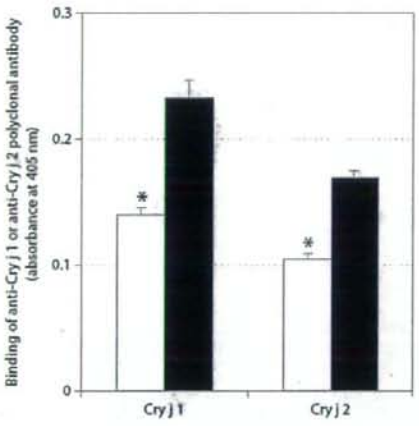

Fig. 2. Reduction in antigenicities of Cry j 1 and Cry j 2 in an ion-irradiated JCP extract. Changes in amounts of Cry j 1 and Cry j 2 recovered in Petri dishes after exposure of JCP allergen to plasma cluster ions (□) or without plasma cluster ions (■) were measured by ELISA using corresponding polyclonal antibodies. The experiment was repeated 3 times. * $p < 0.01$ (paired t test).

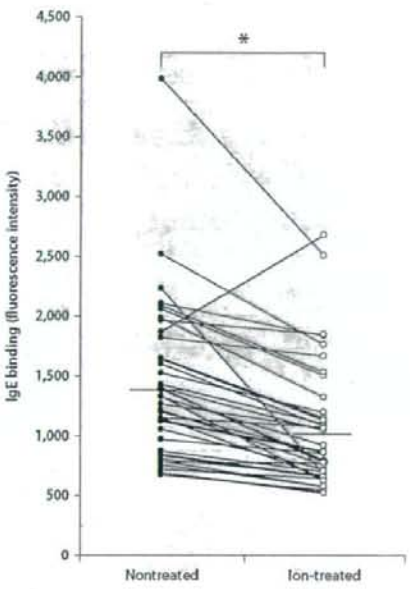

Fig. 3. Decrease in the ability of ion-treated allergens to bind IgE from patients allergic to JCP. IgE binding of ion-treated (○) and nontreated (●) allergens to sera IgE from patients (n = 40) sensitized to JCP was measured by ELISA. * $p < 0.0001$ (Wilcoxon signed-rank test).

ly impaired the IgE binding activity in patients with Japanese cedar pollinosis in contrast with nontreated JCP extract (Wilcoxon signed-rank test, $p < 0.0001$, fig. 3). Furthermore, IgE binding was significantly diminished in 25 out of 40 samples (paired t test, $p < 0.05$, data not shown). SDS-PAGE analysis revealed that ion exposure induced protein degradation in the JCP extract (fig. 4).

To quantitatively evaluate the deterioration of IgE-binding ability, ELISA inhibition was carried out (fig. 5). Reaction of pooled sera IgE from patients with Japanese cedar pollinosis to nontreated JCP extract was completely inhibited by increasing concentrations of nontreated or ion-treated JCP extracts. The concentrations of the competitors, nontreated and ion-treated JCP extracts, required for 50% inhibition of IgE binding were estimated to be 8.4 and 40.5 ng, respectively. Thus, about 80% reduction of IgE binding was observed after exposure of the JCP extract to plasma cluster ions.

Decrease in in vivo Allergenicities of JCP Extract Treated with Plasma Cluster Ions

To compare in vivo allergenic activity between ion-treated JCP and nontreated JCP extracts, intracutaneous reactivity was tested with 10 ng/20 μl using patients allergic to JCP. The intracutaneous tests indicated that the allergenicity of the ion-treated JCP extracts decreased drastically from +++ to + in 8 subjects, from +++ to ± in 1 subject, and from ++ to + in 1 subject, compared to those of nontreated JCP extracts (table 1, fig. 6).

Table 1 also shows the result of the conjunctival reaction tests in the same patients. Ion-treated or nontreated JCP extract solutions (25 ng of protein/5 μl) were dropped into either eye. The scores of conjunctival reaction after the ion treatment indicated that none of the patients tested experienced congestion.

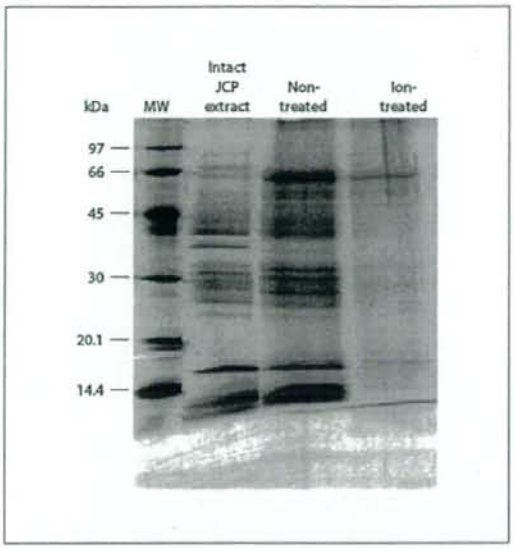

Fig. 4. Deterioration of protein bands in JCP extract by exposure to plasma cluster ions. Ion-treated and nontreated JCP extracts (50 μg as protein) were run on a 15% SDS gel under reduced conditions. Respective protein bands were detected by silver staining.

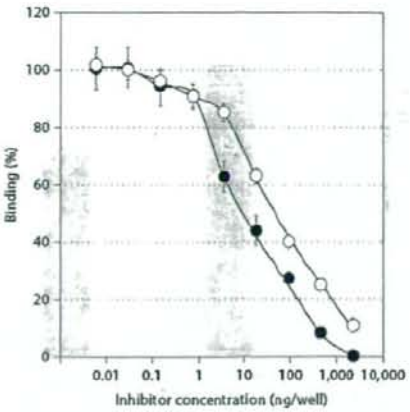

Fig. 5. Inhibition of JCP-allergic IgE binding to nontreated JCP extract with various concentrations of ion-treated or nontreated JCP extract. Pooled sera from patients allergic to JCP were preincubated with increasing concentrations of ion-treated (○) or nontreated (●) JCP extract. The incubation mixture was transferred to a well precoated with intact JCP extract. Then, IgE concentration bound to the well after the competition was determined by ELISA. Each point indicates the mean value from three independent wells and SD value is expressed by the error bar.

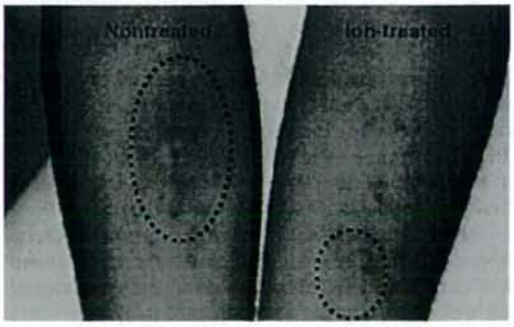

Fig. 6. Intracutaneous testing on forearms of JCP-allergic patients. Aliquots (10 ng/20 μl) obtained after treatment with or without plasma cluster ions were injected intracutaneously. Patient A's left and right forearms show the results of intracutaneous tests using ion-treated (right) and nontreated (left) samples.

Discussion

Nojima and Nishikawa [31] reported that ion treatment for 15–60 min could abolish the proliferating ability of *Cladosporium* sp. spores and, from the disappearance of infectivity and hemagglutination reaction testing, might induce denaturation or decomposition of hemagglutinin on the surface of the virus [29]. Based on this evidence, we anticipated that ion treatment might be capable of lowering practical allergen levels to less than the threshold values eliciting allergic symptoms or sensitizations, because subtractive downmodulation of IgE-binding activity of ion-treated allergens would be dominant among three possibilities of their biochemical alterations, i.e. (1) inactivation or destruction of epitope structures, (2) formation of new epitopes, or (3) improved access of previously hidden epitopes [36–40].

Table 1. Skin test and conjunctival test of the ion-treated and nontreated JCP extracts

Patient	RAST (score)	Skin test[1]		Conjunctival test[2]	
		ion-treated	non-treated	ion-treated	non-treated
A	5.78 (3)	+	+++	–	+
B	21.8 (4)	+	+++	–	+
C	64.0 (5)	+	+++	–	+
D	5.08 (3)	+	+++	–	–
E	50.4 (5)	+	+++	–	+
F	20.5 (4)	+	+++	–	+
G	80.7 (5)	+	+++	–	+
H	34.4 (4)	+	++	–	+
I	35.6 (4)	±	+++	–	+
J	34.1 (4)	+	+++	–	+

[1] Skin tests were conducted by injecting intracutaneously 20 μl of solution containing the ion-treated or nontreated JCP extracts (10 ng) dissolved in 0.9% NaCl. For erythema of 0–9, 10–19, 20–29, 30–39 or >40 mm in mean diameter, appearing after 15 min, a score of –, ±, +, ++ or +++ was assigned, respectively.

[2] One lower eyelid of each patient allergic to JCP was treated with a drop of a sample solution (25 ng/5 μl in 0.9% NaCl). After 15 min, the conjunctiva was compared with the control eye to examine for congestion. The symbols of –, +, ++, or +++ were assigned for the degree of congestion as follows: no, localized, intermediate, and strong, respectively.

In accord with a report that a sufficient amount of JCP capable of provoking allergenic symptoms was present even in house dust before the pollen season [41], JCP was selected as our first choice of airborne allergens. Due to the inherent aerodynamic characteristics of JCP with a particle size of 30–37 μm, we were confronted with great difficulties in dispersing and floating JCP uniformly in a chamber. Indeed, JCP fell onto the bottom collecting dish as quickly as 2 s without wafting in the air under our experimental conditions. Similarly, a troublesome problem regarding a uniform suspension of powdered JCP extract also occurred. Accordingly, we could not help but use an atomized JCP extract as our next best choice in place of JCP. Further, the experiment using a nebulized JCP extract was considered to serve as a model reasonably similar to the actual phenomenon, because we could not recognize any remarkable differences in the reactions of plasma cluster ions to either wafted or atomized JCP extracts, although floated allergens would probably not be surrounded by liquid, since plasma cluster ions consist of positive and negative ions containing a cluster of water molecules.

The molecular structures of the plasma cluster ions generated were identified as $H_3O^+(H_2O)_m$ and $O_2^-(H_2O)_n$ ($m, n \geq 0$) as the major ions [31, 42], while ozone and the other ions were negligible [29–31]. The rate of decrease in in vitro antigenic activities (fig. 3, 4) and in in vivo allergenic activities (table 1) was proportional to the densities of the plasma cluster ions caused by changing the input voltage. As a plausible explanation for the reduction in the antigenicity and allergenicity of JCP extract by treatment with plasma cluster ions, it is possible that the cleavage of various proteins by hydroxyl radicals, probably produced by collisions among cationic and anionic cluster ions, would occur because ion irradiation did not produce WST-1 formazan from a WST-1 solution, but generated formazan in the presence of H_2O_2 in a sealed dark reaction vessel and did not convert Amplex Red to resorufin in the presence of peroxidase (data not shown). Moreover, anionic cluster ions alone hardly reduced the antigenicity and allergenicity of JCP extract during short-term (90 s) contact.

Pollen and house dust mite and cockroach allergens only become airborne after vigorous disturbance and then settle rapidly [16]. Environmental modifications to limit exposure to these allergens should have significant potential to abate the prevalence of allergic diseases. Chemical treatments exhibited only temporarily beneficial effects against reservoirs of allergens but less against airborne allergens. Consequently, repeated application or additional approaches are required to control the bulk of allergens found in residences [18–21, 43]. Physical treatments seem more suitable against airborne allergens. HEPA filter cleaners have been shown to effectively remove airborne allergens; however, these devices were also shown to be ineffective in facilitating asthma control [22]. Airborne allergen avoidance using a HEPA filter has been similarly investigated with inconclusive and sometimes contradictory results [22–24].

Previous research on the use of negative ion generators that electrostatically precipitated airborne dust and allergens has shown little or no evidence of any clinical benefits from their use [25]. Warner et al. [26] reported that the use of ionizers could not be recommended in homes of asthmatic subjects to improve their symptoms, but that a significant abatement of airborne allergen concentrations may be useful as part of an allergen avoidance regimen. Ionizers and/or air filtration units did not reduce the levels of mite allergens in indoor environments, since the largest amounts of these allergens remained in settled dust [44]. The development of the most effective way to avoid becoming sensitized and provoking allergic symp-

toms would be to create the ideal circumstances of getting rid of all allergens from our living environments. Recently, Goodman and Hughes [27, 28, 45] developed a corona discharge ionizer and observed up to a 92% reduction in the concentration of settled Der p 1 in the presence of 2.40 ppm ozone for 3 weeks in an unoccupied but furnished office room. However, milder means to decrease allergen concentrations and so maintain the quality of daily life remain to be developed.

The significance of the results presented here is that the decrease in antigenicity and allergenicity of allergen can be achieved even by short-term (90 s) contact between nebulized JCP extract and plasma cluster ions. Recently, Takahashi et al. [46] reported that orbicles of 0.6- to 1.2-μm size reside in a pollen-adhered form or a free form in quantities more than 8 times those of the pollen numbers scattering from the anthers. As it is conceivable from the particle size that an orbicle containing Cry j 1 would waft easily into a living environment, an orbicle might be more important in the induction of allergic symptoms and more effective in its response to ion treatment than pollen.

The use of plasma cluster ions has much potential to inactivate the antigenicity and allergenicity of various airborne allergens without an elevation of ozone concentrations in domestic surroundings. From our preliminary results, we observed a similar decrease in specific IgE-binding activity to house dust mite allergens that became airborne after vigorous disturbance, from exposure to plasma cluster ions (3,000 counts/ml) for 30 min (data not shown). Further, the safety of successive exposures to the ions has been confirmed. Further research will be necessary to confirm the benefits, especially clinical ones, of plasma cluster ions under natural conditions in domestic environments.

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References

1 O'Connell EJ: Pediatric allergy: a brief review of risk factors associated with developing allergic disease in childhood. Ann Allergy Asthma Immunol 2003;90:53–58.

2 Nicolai T: Pollution, environmental factors and childhood respiratory allergic disease. Toxicology 2002;181–182:317–321.

3 Platts-Mills TA, Blumenthal K, Perzanowski M, Woodfolk JA: Determinants of clinical allergic disease. The relevance of indoor allergens to the increase in asthma. Am J Respir Crit Care Med 2000;162:S128–S133.

4 Saito Y: Japanese cedar pollinosis (in Japanese). Infect Inflamm Immun 1995;25:54–60.

5 Okuda M: Overcoming Japanese cedar pollinosis (in Japanese). Med Asahi 2002; 31(suppl):1–6.

6 Yasueda H, Yui Y, Shimizu T, Shida T: Isolation and partial characterization of major allergen from Japanese cedar *(Cryptomeria japonica)* pollen. J Allergy Clin Immunol 1983; 71:77–86.

7 Sone T, Komiyama N, Shimizu K, Kusakabe T, Morikubo K, Kino K: Cloning and sequencing of cDNA coding for Cry j I, a major allergen of Japanese cedar pollen. Biochem Biophys Res Commun 1994;199:619–625.

8 Taniguchi Y, Ono A, Sawatani M, Nanba M, Kohno K, Usui M, Kurimoto M, Matuhasi T: Cry j I, a major allergen of Japanese cedar pollen, has pectate lyase enzyme activity. Allergy 1995;50:90–93.

9 Sakaguchi M, Inouye S, Taniai M, Ando S, Usui M, Matuhashi T: Identification of the second major allergen of Japanese cedar pollen. Allergy 1990;45:309–312.

10 Komiyama N, Sone T, Shimizu K, Morikubo K, Kino K: cDNA cloning and expression of Cry j II, the second major allergen of Japanese cedar pollen. Biochem Biophys Res Commun 1994;201:1021–1028.

11 Namba M, Kurose M, Torigoe K, Hino K, Taniguchi Y, Fukuda S, Usui M, Kurimoto M: Molecular cloning of the second major allergen, Cry j II, from Japanese cedar pollen. FEBS Lett 1994;353:124–128.

12 Ohtsuki T, Taniguchi Y, Kohno K, Fukuda S, Usui M, Kurimoto M: Cry j 2, a major allergen of Japanese cedar pollen, shows polymethylgalacturonase activity. Allergy 1995; 50:483–488.

13 Kawamoto S, Fujimura T, Nishida M, Tanaka T, Aki T, Masubuchi M, Hayashi T, Suzuki O, Shigeta S, Ono K: Molecular cloning and characterization of a new Japanese cedar pollen allergen homologous to plant isoflavone reductase family. Clin Exp Allergy 2002;32:1064–1070.

14 Fujimura T, Shigeta S, Suwa T, Kawamoto S, Aki T, Masubuchi M, Hayashi T, Hide M, Ono K: Molecular cloning of a class IV chitinase allergen from the Japanese cedar *(Cryptomeria japonica)* pollen and competitive inhibition of its IgE-binding capacity by latex C-serum. Clin Exp Allergy 2005;35: 234–243.

15 Fujimura T, Shigeta S, Kawamoto S, Aki T, Masubuchi M, Hayashi T, Yoshizato K, Ono K: Two-dimensional IgE-binding spectrum of Japanese cedar *(Cryptomeria japonica)* pollen allergens. Int Arch Allergy Clin Immunol 2004;133:125–135.

16 Custovic A, Simpson A, Chapman MD, Woodcock A: Allergen avoidance in the treatment of asthma and atopic disorders. Thorax 1998;53:63–72.

17 Tovey E, Marks G: Methods and effectiveness of environmental control. J Allergy Clin Immunol 1999;103:179–191.

18 Green WF, Nicholas NR, Salome CM, Woolcock AJ: Reduction of house dust mite allergens: effective of spraying carpets and blankets with Allersearch DMS, an acaricide combined with an allergen reducing agent. Clin Exp Allergy 1989;19:203–207.

19 Tovey E, Marks G, Matthew M, Green W, Woolcock A: Changes in mite allergen Der p 1 in house dust following spraying with a tannic acid/acaricide solution. Clin Exp 1992;22:67–74.

20 Woodfolk JA, Hayden ML, Couture N, Platts-Mills TA: Chemical treatment of carpets to reduce allergen: comparison of the effects of tannic acid and other treatments on proteins derived from dust mites and cats. J Allergy Clin Immunol 1995;96:325–333.

▶21 Lau S, Wahn J, Schulz G, Sommerfeld C, Wahn U: Placebo-controlled study of the mite allergen-reducing effect of tannic acid plus benzyl benzoate on carpets in homes of children with house dust mite sensitization and asthma. Pediatr Allergy Immunol 2002; 13:31–36.

▶22 Popplewell EJ, Innes VA, Lloyd-Hughes S, Jenkins EL, Khdir K, Bryant TN, Warner JO, Warner JA: The effect of high-efficiency and standard vacuum-cleaners on mite, cat and dog allergen levels and clinical progress. Pediatr Allergy Immunol 2000;11:142–148.

▶23 Francis H, Fletcher G, Anthony G, Pickering C, Oldham L, Hadley E, Custovic A, Niven R: Clinical effects of air filters in homes of asthmatic adults sensitized and exposed to pet allergens. Clin Exp Allergy 2003;33:101–105.

▶24 Gore RB, Durrell B, Bishop S, Curbishley L, Woodcock A, Custovic A: High-efficiency particulate arrest-filter vacuum cleaners increase personal cat allergen exposure in homes with cats. J Allergy Clin Immunol 2003;111:784–787.

▶25 Nogrady SG, Furnass SB: Ionisers in the management of bronchial asthma. Thorax 1983;38:919–922.

▶26 Warner JA, Marchant JL, Warner JO: Double-blind trial of ionizers in children with asthma sensitive to the house dust mite. Thorax 1993;48:330–333.

▶27 Goodman N, Hughes JF: The effect of corona discharge on Der p 1. Clin Exp Allergy 2002; 32:515–519.

▶28 Goodman N, Hughes JF: Long-range destruction of Der p 1 using experimental and commercially available ionizers. Clin Exp Allergy 2002;32:1613–1619.

29 Nishikawa K, Nojima H, Aoki M, Kuroda Y: Development of novel air purification technology using ions generated by discharge plasma. ii. Inactivation of influenza virus in air. Healthy Buildings 2003 7th International Conference, Singapore, 2003, pp 660–665.

▶30 Nishikawa K, Nojima H: Air purification effect of positively and negatively charged ions generated by discharge plasma at atmospheric pressure. Jpn J Appl Phys 2001;40: L835–L837.

31 Nojima H, Nishikawa K: Air purification technology using ions generated by discharge plasma at atmospheric pressure (in Japanese). J Inst Electrostatics Jpn 2002;26: 153–157.

▶32 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–275.

▶33 Aki T, Kodama T, Fujikawa A, Miura K, Shigeta S, Wada T, Jyo T, Murooka Y, Oka S, Ono K: Immunochemical characterization of recombinant and native tropomyosins as a new allergen from the house dust mite, *Dermatophagoides farinae*. J Allergy Clin Immunol 1995;96:74–83.

▶34 Yamaguchi T, Tanaka S, Shigeta S, Wada T, Tsuboi S, Otsuka T, Katsutani T, Jyo T, Oka S, Ono K: A low-molecular-size LM2 allergen in mite, *Dermatophagoides farinae*, extracts containing feces which elicits conjunctival reactions in patients with bronchial asthma. Jpn J Allergol 1994;43: 815–824.

▶35 Yamaguchi T, Tanaka S, Shigeta S, Wada T, Tsuboi S, Otsuka T, Katsutani T, Jyo T, Oka S, Ono K: Low-molecular-size allergens, LM-1s, in feces extract of *Dermatophagoides farinae* which elicit histamine release from washed blood cells of patients with bronchial asthma. Jpn J Allergol 1994;43:701–710.

▶36 Kusunoki T, Inoue Y, Korematsu S, Harazaki M, Yokota T, Hosoi S: Comparison of skin prick test with serially diluted wild-type and genetically engineered recombinant Der f 2. Ann Allergy Asthma Immunol 2000;84: 366–368.

▶37 Korematsu S, Tanaka Y, Hosoi S, Koyanagi S, Yokota T, Mikami B, Minato N: C8/119S mutation of major mite allergen Derf-2 leads to degenerate secondary structure and molecular polymerization and induces potent and exclusive Th1 cell differentiation. J Immunol 2000;165:2895–2902.

38 Davis PJ, Williams SC: Protein modification by thermal processing. Allergy 1998;53(46 suppl):102–105.

▶39 Rytkonen J, Karttunen TJ, Karttunen R, Valkonen KH, Jenmalm MC, Alatossava T, Bjorksten B, Kokkonen J: Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats: denatured betaLG induces a more intensive local immunologic response than native betaLG. Pediatr Allergy Immunol 2002;13:269–277.

▶40 Mine Y, Zhang JW: Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. J Agric Food Chem 2002;50:2679–2683.

▶41 Enomoto T, Ohnishi S, Dake Y, Sakoda T, Saitoh Y, Sogo H, Seno S, Fujiki Y, Fujumura S: Japanese cedar pollens in house dust (in Japanese). Jpn J Allergol 2001;50:535–539.

42 Yagi H, Nishikawa K, Nojima H: Japan Kokai Tokkyo Koho, patent 2003-201722.

▶43 Codina R, Lockey RF, Diwadkar R, Mobly LL, Godfrey S: Disodium octaborate tetrahydrate (DOT) application and vacuum cleaning, a combined strategy to control house dust mites. Allergy 2003;58:318–324.

▶44 Liccardi G, Custovic A, Cazzola M, Russo M, D'Amato M, D'Amato G: Avoidance of allergens and air pollutants in respiratory allergy. Allergy 2001;56:705–722.

▶45 Goodman N, Hughes JF: The effect of corona discharge on dust mite and cat allergens. J Electrostatics 2004;60:69–91.

▶46 Takahashi Y, Sasaki K, Nakamura S, Miki-Hiroshige H, Nitta H: Aerodynamic size distribution of the particles emitted from the flowers of allergologically important plants. Grana 1995;34:45–49.

Crucial Commitment of Proteolytic Activity of a Purified Recombinant Major House Dust Mite Allergen Der p1 to Sensitization toward IgE and IgG Responses[1]

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The major proteolytic allergen derived from the house dust mite *Dermatophagoides pteronyssinus*, Der p1, is one of the most clinically relevant allergens worldwide. In the present study, we evaluate the contribution of the proteolytic activity and structure of a highly purified rDer p 1 to immune responses. Mice were i.p. immunized with three forms of rDer p 1 adsorbed to Alum: one enzymatically active, one treated with an irreversible cysteine protease-specific inhibitor, E-64, and one heat denatured. Immunization with E-64-treated or heat-denatured rDer p 1 elicited much less production of serum total IgE and not only rDer p 1-specific IgE but also IgGs compared with immunization with active rDer p 1. Assays for Ab-binding and its inhibition and structural analyses indicated that E-64-treated rDer p 1 retained its global structure and conformational B cell epitopes. A proliferative response and production of IL-5 by spleen cells restimulated with rDer p 1 were observed on immunization with the active rDer p 1 but not E-64-treated rDer p 1. The cells from mice immunized with heat-denatured rDer p 1 exhibited the highest levels of proliferation and production of IL-5 and IFN-γ. The results indicate that the proteolytic activity of the highly purified rDer p 1 crucially commits to the sensitization process, including both IgE and IgG responses. Additionally, we demonstrated immunogenic differences by functional or structural manipulations of the rDer p 1. The findings have implications for sensitization to this relevant allergen in humans and for the design of modified allergen-vaccines for future allergen-specific immunotherapy. *The Journal of Immunology*, 2006, 177: 1609–1617.

House dust mites of two species, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are major sources of allergens associated with allergic diseases such as asthma, rhinitis, and atopic dermatitis (1–4). Group 1 and group 2 allergens are major allergens derived from house dust mites based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract (4–10). The group 1 allergens, Der p 1 from *D. pteronyssinus* and Der f1 from *D. farinae*, belong to the papain-like cysteine protease family (11, 12) and actually exhibit cysteine protease activity (13–16). The cDNAs for Der p 1 and Der f 1 were isolated when cDNA cloning of allergens began (11, 12, 17). They encode signal peptides of 18-aa residues responsible for secretion, 80-residue propeptides, and 222-residue mature portions for Der p 1 and 223 residues for Der f 1. Mature Der p 1 and Der f 1 have 82% sequence identity with each other. At least 12 isoforms with different amino acid sequences for Der p 1 (18) and two for Der f 1 (12, 19) have been identified. Very recently, the crystal structure of a recombinant mutant protein of the Der p 1 precursor was determined (20).

The cysteine protease activity of Der p 1 and Der f 1 has been suggested to be involved in the pathogenesis of allergies by facilitating the passage of their own and other allergens across the epithelium (21, 22), cleaving and/or interacting with cell surface molecules and intrinsic protease inhibitors (23–30), and modulating the function of various cells (23, 27, 31–39). Herbert et al. (21) speculated that cysteine protease activity of natural Der p 1 could be activated in vivo by glutathione in the airway on the basis of their in vitro observations. However, which molecule(s) and/or which cell type(s) are the most critical targets of the cysteine protease activity of Der p 1 and Der f 1 for pathogenesis of allergy and where Der p 1 and Der f 1 become active and work effectively are not well investigated. Two research groups reported an enhancement of IgE production (40, 41) and induction of Th2 responses (42) by the proteolytic activity of Der p 1 in mice, respectively. Gough et al. (40, 41) reported that treatment of Der p 1 with an irreversible cysteine protease-specific inhibitor, E-64, partially reduced the IgE-eliciting activity of the allergen without affecting production of IgG in mice.

Recently, we have succeeded in preparation of rDer p 1 and rDer f 1, which are similar to their natural counterparts in allergenicity, structure, and m.w. (43–46), have proteolytic activities (44–46), show a similar substrate-specificity for cysteine protease activity as the natural types (16, 28), and are free from mite-derived serine proteases (16). They exhibited enzymatic activity in vitro to cleave human CD23 and CD25 on the cell surface and human α1-antitrypsin, which had been reported to be cleaved by natural Der p 1 and IgE-eliciting activity in vivo in mice similar to natural Der p

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1 and Der f 1 (28). The highly purified and fully active rDer p 1 and rDer f 1 will be the basis for allergen standardization and the design of safer and more effective allergen-vaccines and be useful for studies in vitro and in vivo to investigate the roles of their function and structure in allergic diseases.

Disruption or modification of the tertiary structure of an allergen is an efficient way to reduce the IgE-binding activity of that allergen when designing safer vaccines for allergen-specific immunotherapy (17, 47–50). In the case of Der p 1, as it modulates biological responses through its biochemical function, proteolytic activity, we considered that the function also could be a target for therapy when designing a novel allergen-vaccine using its recombinant form. In the present study, to obtain information for the modulation of immune responses by changing the biochemical function or tertiary structure of the rDer p 1, we compare immune responses including IgE-eliciting activity in mice immunized with three types of rDer p 1, one enzymatically active, one treated with the protease inhibitor, and one heat denatured. We demonstrate that the efficient in vivo responses, including production of not only IgE but also IgG against the highly purified rDer p 1, are crucially dependent on the cysteine protease activity in mice and that the three types of rDer p 1 differing in function or structure elicit distinctly different immune responses.

Materials and Methods

Recombinant Der p 1

The amino acid sequence of the pre-precursor form of Der p 1 (pro-Der p 1)[3] clone selected for expression was identical with Der p 1.0102 (18), which was reported previously as "clone (c)" by Chua et al. (51), except for one residue (−95) in the signal sequence (44). For the immunization of mice, we used a recombinant mutant Der p 1 designated Der p 1-N52Q, which has the same m.w. as natural Der p 1 (43, 44). Protein concentrations were determined by means of the Bradford procedure with a protein assay kit (Bio-Rad). LPS levels of the purified Der p 1-N52Q were shown to be <20 ng-LPS/mg-protein by using Endospecy (Seikagaku). As B cell epitopes for Der p 1-N52Q and Der p 1-WT are considered to be equivalent (43), we used the hyperglycosylated Der p 1-WT for the coating of ELISA plates to detect Der p 1-specific Abs as an alterative to Der p 1-N52Q, production level of which in yeast is much lower than that of Der p 1-WT (44, 52), although Der p 1-N52Q was used for coating plates in Figs. 4 and 5*A*. Der p 1-N52Q is described as rDer p 1 throughout this article.

Proteolytic activity

Cysteine protease activity was measured as described previously (16).

Immunization of mice

Six- to 8-wk-old female CBA/J mice were purchased from Charles River Japan and were maintained in a specific pathogen-free animal facility at Juntendo University throughout the study. Before use, 100 μM rDer p 1 was incubated with 5 mM L-cysteine (37°C, 15 min). Then the enzymatic activity was abolished by addition of E-64 (Peptide Institute) (37°C, 30 min) or by heating (98°C, 30 min). The treatment with E-64 was performed with 62.5 μM activated rDer p 1, 3.13 mM L-cysteine, and 1.56 mM E-64 in PBS, and then, the Ag was diluted with PBS. OVA (grade V; Sigma-Aldrich) and E-64-treated OVA were prepared as described above as controls. The heat denaturation was performed with 10 μM activated rDer p 1 and 0.5 mM L-cysteine after dilution with PBS. The Ags were incubated with Alum (ImjectAlum; Pierce) and further diluted to appropriate volumes with saline. Mice were given four weekly i.p. injections of 2.5 μg of each Ag (28). Peripheral blood was collected weekly from the retroorbital plexus until 1 wk after the last injection. All sera were stored at −20°C before analysis for Ab content.

ELISA for total IgE and levels of Ag-specific Abs

Serum total IgE was measured by a sandwich ELISA as described previously (28). Ag-specific Abs were detected on plates coated with a 10 μg/ml solution of Ags and blocked with BlockAce (Snow Brand) and developed with biotinylated or HRP-conjugated anti-mouse IgE mAb (clone LO-ME-2; Serotec), HRP-conjugated anti-mouse IgG1 mAb (clone X56; BD Biosciences), HRP-conjugated goat anti-mouse IgG2a Ab (Southern Biotechnology Associates), and HRP-conjugated rabbit anti-mouse IgG2b Ab (Zymed Laboratories). HRP-conjugated Extravidine (Sigma-Aldrich) was used in conjunction with biotinylated anti-mouse IgE mAb for detection of Der p 1-specific IgE. Serum dilutions were 1/10, 1/15,000, 1/100, and 1/100 for detecting Ag-specific IgE, IgG1, IgG2a, and IgG2b, respectively. For detecting Der p 1- or OVA-specific IgE, the diluted sera were depleted of IgGs (40) as follows. Sera diluted with PBS containing 0.05% (v/v) Tween 20 (PBST) and 10% (v/v) BlockAce were depleted of IgG with protein G-Sepharose 4FF (Amersham Biosciences) within wells of round-bottom microtiter plates (Corning) with agitation at a speed of 1000 rpm by a Bio-Shaker (Titec) for 30 min at room temperature. The total volume of the suspension, the volume of protein G beads, the volume of original serum added, and the final serum dilution were 78, 18, and 6 μl/well and 1/10, respectively. After centrifugation with a swing rotor at 1900 × *g* for 2 min, the supernatants were used for assays. Volumes of 50 μl from the supernatants in the wells were moved to wells on other plates, which were coated with allergens and blocked as described above. The dilution of the second Abs and the HRP-conjugated Extravidine were 1/1000 and 1/500, respectively. Binding of the conjugates was visualized and measured as described previously (28).

Sensitive ELISA for Ag-specific IgE and titration of Ag-specific Abs

A more sensitive ELISA for Ag-specific IgE was performed using the commercially available immunoreaction enhancer solutions, CanGetSignal (TOYOBO) (53, 54), without the removal of IgG was performed as follows. Volumes of 50 μl of sera diluted with solution 1 of CanGetSignal were added to wells on plates, which were coated with allergens and blocked as described above. After incubation for 15 h at 4°C and three washes with PBST, HRP-conjugated anti-mouse IgE mAb (clone LO-ME-2; Serotec) diluted with solution 2 of CanGetSignal was added to the plates (1/2000 dilution, 50 μl/well). After incubation for 5 h at room temperature and three washes with PBST, the color was developed for 20 min using tetramethyl benzidine (100 μl/well) (BD-OptEIA kit; BD-biosciences), and the reaction was stopped by adding 2 N sulfuric acid (50 μl/well). The OD at 450 nm, from which that at 570 nm was subtracted, was used as the signal for allergen-specific IgE.

For titration, Ag-specific IgGs were also detected, as well as Ag-specific IgE, with the modification that incubation with the sera and that with the secondary Abs was for 90 min at 37°C. The Ag-specific Ab titers were identified as the reciprocal of the dilutions giving an absorbance of 0.1 calculated from titration curves by using Prism version 4.0 (GraphPad).

Inhibition assay

A pooled serum, which was collected from mice immunized with activated rDer p 1 and diluted (1/20,000) with PBST containing and 10% (v/v) BlockAce, was preincubated with serially diluted inhibitors (rDer p 1 or E-64-treated rDer p 1 dialyzed against PBS) for 30 min at room temperature and then added to plates, which were coated with rDer p 1 or E-64-treated rDer p 1 and blocked. The plates were incubated for 15 h at room temperature. After three washes with PBST, the plates were incubated with HRP-conjugated anti-mouse IgG1 mAb diluted (1/2000) with solution 2 of CanGetSignal for 5 h at 37°C. Binding of the conjugate was visualized and measured. The percentage of inhibition was expressed as the relative reduction of the absorbance in each sample to that when no inhibitors were added.

Circular dichroism

Activated rDer p 1 (125 μg/ml) and the rDer p 1 treated with E-64 were subjected to measurements of circular dichroism spectra after dialysis as described previously (43). Ellipticities at 190–260 nm after smoothing are shown.

Molecular modeling

A model of the Der p 1/E-64 complex was made by superimposing the Protein Data Bank entries 1XKG (a mutant of pro-Der p 1) (20) and 1ATK (cathepsin K/E-64 complex) (55) and removing unwanted portions. The figure was made by PyMOL (DeLano Scientific).

Splenocyte responses

Spleens were aseptically removed 1 wk after the last immunization. Cell suspensions were prepared by gently teasing apart the tissue in RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 10% (v/v)

[3] Abbreviation used in this paper: pro-Der p 1, precursor form of Der p 1.

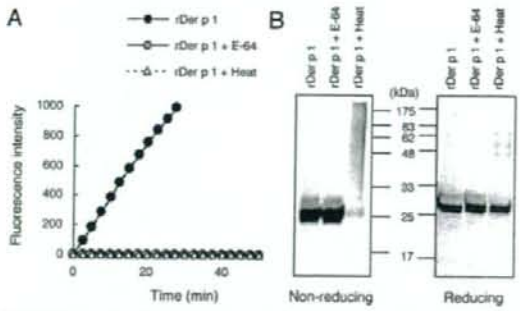

FIGURE 1. Inactivation of the proteolytic activity of rDer p 1 by E-64 or heating and polymerization of heat-denatured rDer p 1 with intermolecular disulfide bond formation. *A*, Proteolytic activity. The rDer p 1 derivatives were treated with DTT before the reaction. Time courses of digestion of a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA, were shown. *B*, SDS-PAGE. The samples were boiled in the absence (nonreducing) or presence of 2-ME (reducing) in the sample buffer, subjected to SDS-PAGE (15–25%), and silver stained.

heat-inactivated FCS, 0.05 mM 2-ME, and antibiotics. Following homogenization by passage through stainless steel mesh and centrifugation at 500 × *g* for 5 min at 4°C, cells were resuspended in 1 ml of ACK buffer (8.3 g of NH_4Cl, 1 g of K_2HCO_3, and 37.2 mg of EDTA-2Na in 1 liter) at room temperature for 5 min to deplete RBC. After dilution with 10 ml of the medium, the cell suspensions were centrifuged, resuspended in 10 ml of the medium, centrifuged, and resuspended in 3 ml of the medium at 4°C. Viable cells were enumerated by trypan blue exclusion and the suspensions adjusted to 4×10^6 cells/ml. Aliquots of 100 μl of medium containing rDer p 1 (20 μg/ml) or medium alone were added to 96-well, flat-bottom, tissue culture plates (Corning), and 100-μl aliquots of the cell suspension were added. To determine cytokine production, culture supernatants were collected at 72 h and subjected to ELISA using kits (Genzyme Techne).

For estimating proliferative responses, the cultures in triplicate wells were pulsed with 1 μCi/well [^{3}H]thymidine (PerkinElmer Japan) for the next 16 h and harvested using a Micro 96 Harvester (Skatoron). Incorporated radioactivity was measured in a microbeta counter (MicroBeta Plus; Wallac). Data are shown as stimulation indexes that were calculated as the ratio of the counts per minute of culture wells containing rDer p 1 to that of wells containing medium alone. The mean of triplicate wells was used as the data for one mouse.

Statistical analysis

Student's *t* test (two-tailed) was used to evaluate the significance of the differences, although the Mann-Whitney *U* test was used to analyze the Ab titration data. A value of $p < 0.05$ was regarded as statistically significant. Data shown are representative of three independent immunization experiments.

Results

Inactivation of proteolytic activity of rDer p 1 by E-64 or heating

By an assay using a synthetic fluorogenic substrate, we confirmed that the proteolytic activity of the E-64-treated rDer p 1 and heat-denatured rDer p 1 was inactivated (Fig. 1*A*). On SDS-PAGE, rDer p 1 and E-64-treated rDer p 1 showed single bands of similar mobility (Fig. 1*B*). Their mobilities were higher in nonreducing conditions on SDS-PAGE than in reducing conditions, indicating molecular compactness due to the formation of intramolecular disulfide bonds. The two also showed similar elution volumes in size exclusion column chromatography (our unpublished observation). The results indicated that E-64-treated rDer p 1 remained monomeric. On the other hand, heat-denatured rDer p 1 showed a broad-ranged smear band of high m.w. under nonreducing conditions while a major band with a mobility equivalent to rDer p 1 and E-64-treated rDer p 1 was detected under reducing conditions (Fig. 1*B*), indicating that the heat-denatured rDer p 1 was polymerized by the formation of intermolecular scrambled disulfide bonds after disruption of the correct intramolecular disulfide bonds by heating in the presence of L-cysteine.

E-64-treated rDer p 1 and heat-denatured rDer p 1 elicited much less production of total IgE and Der p 1-specific Abs compared with active rDer p 1

While mice immunized with the enzymatically active rDer p 1 produced total IgE (Fig. 2*A*, *left*) and Der p 1-specific IgE (Fig. 2, *B*, *left*, and *C*), those immunized with E-64-treated rDer p 1 and heat-denatured Der p 1 produced as little IgE as control mice and significantly decreased levels of Der p 1-specific IgE. The level of Der p 1-specific IgE at 4 wk was determined to be significantly higher on immunization with active rDer p 1 than on immunization with E-64-treated rDer p 1 by the Mann-Whitney *U* test ($p = 0.0022$) in Fig. 2*B* and more clearly in Figs. 2*C* and 3*A*, where an ELISA with higher sensitivity using immunoreaction enhancer solutions was applied as described in *Materials and Methods*. The total IgE level peaked at 2 wk (Fig. 2*A*, *left*), preceding the elevation in the level of Der p 1-specific IgE (Fig. 2*B*, *left*). Der p 1-specific IgGs were also at significantly low levels on immunization with E-64-treated rDer p 1 and heat-denatured rDer p 1 compared with active rDer p 1 (Figs. 2, *D–F*, *left*, and 3*B*). Compared with active rDer p 1, geometric means of titers for Der p 1-specific IgE, IgG1, IgG2a, and IgG2b decreased to <1/20, ~1/100, <1/15, and ~1/30, respectively, on immunization with E-64-treated rDer p 1 and further on immunization with heat-denatured rDer p 1 (Fig. 3). In terms of the Ab titer following immunization with active rDer p 1, the IgG subclasses tested ranked as follows: IgG1 > IgG2b > IgG2a.

Injection of E-64 with OVA did not affect levels of the Abs (Figs. 2, *A*, *B*, and *D–F*, *right*, and 3), suggesting that the reduction of total IgE and Der p 1-specific Abs on immunization with E-64-treated rDer p 1 was caused by elimination of the proteolytic activity of rDer p 1 and not by a nonspecific effect of excess of E-64 injected with rDer p 1. On OVA immunization, total IgE and OVA-specific IgE levels increased in a time-dependent manner (Fig. 2, *A* and *B*, *right*).

B cell epitopes were maintained in E-64-treated rDer p 1 but disrupted in heat-denatured rDer p 1

In plates coated with E-64-treated rDer p 1 (Fig. 4, immobilized Ag: rDer p 1 + E-64), the Ab-binding pattern was similar to that in rDer p 1-coated plates (Fig. 4, immobilized Ag: rDer p 1), indicating that the B cell epitope structure of rDer p 1 was maintained after E-64-treatment. Der p 1-specific IgGs in sera from rDer p 1-immunized mice failed to bind plates coated with heat-denatured rDer p 1, indicating that the B cell epitopes of rDer p 1 were disrupted by the heating (Fig. 4, immobilized Ag: rDer p 1 + heat). The results indicated that the reduction in the production of Der p 1-specific Abs on immunization with E-64-treated rDer p 1 or heat-denatured rDer p 1 (Figs. 2, *B–F*, and 3) was not due to a failure to detect Abs recognizing structures unique to the modified conformation of E-64-treated rDer p 1 or heat-denatured rDer p 1.

E-64-treated rDer p 1 retained its global structure

The rDer p 1 without and with E-64 treatment completely inhibited each other in the solid phase assay for IgG1 binding at higher concentrations of inhibitors (Fig. 5*A*). E-64-treated rDer p 1 exhibited a similar circular dichroism spectrum to rDer p 1, showing similarity in their secondary structure (Fig. 5*B*). E-64 is a low m.w. irreversible cysteine protease-specific inhibitor and covalently

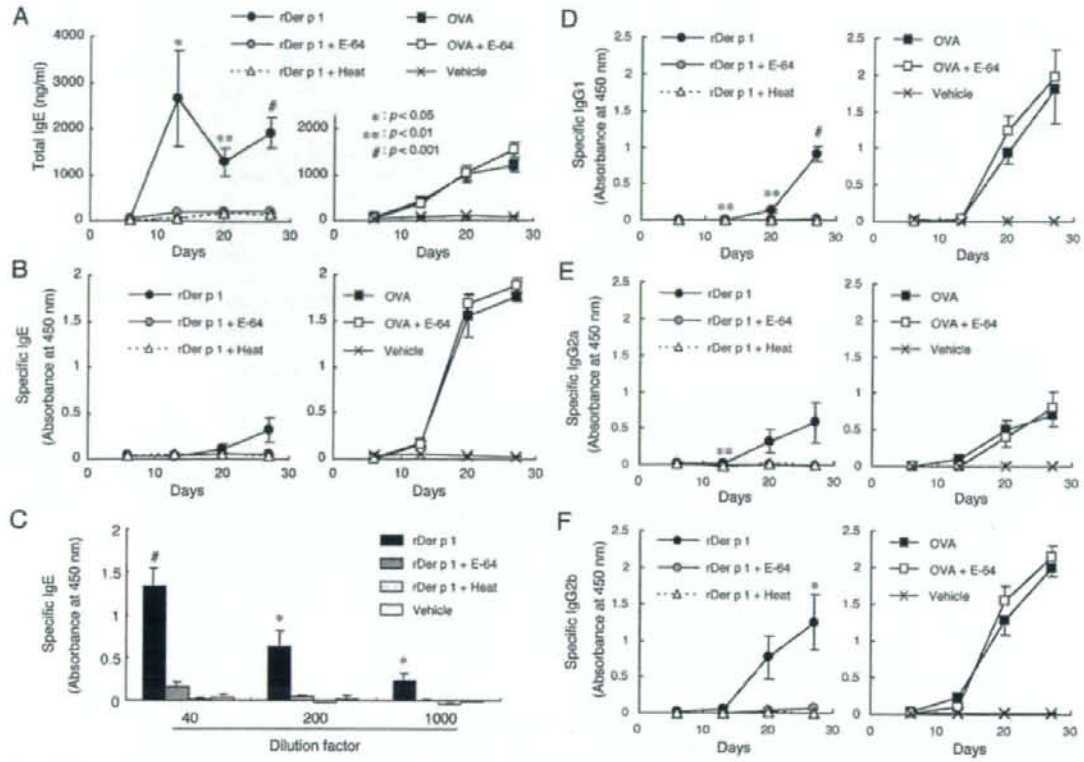

FIGURE 2. Serum Ab production on immunization with rDer p 1 activated with L-cysteine, E-64-treated rDer p 1, heat-denatured Der p 1, OVA, and E-64-treated OVA. The Ags were injected to mice without removal of L-cysteine and E-64. *A*, Total IgE. Der p 1-specific IgE (*B*), IgG1 (*D*), IgG2a (*E*), and IgG2b (*F*) were measured except for OVA and OVA + E-64 (OVA-specific Abs). Serum dilution factors were 10, 15,000, 100, and 100 for detecting Ag-specific IgE, IgG1, IgG2a, and IgG2b, respectively. *C*, Der p 1-specific IgE at 4 wk measured by ELISA with higher sensitivity. 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, and the statistically significant differences were indicated (*, $p < 0.05$; **, $p < 0.01$; and #, $p < 0.001$).

binds to the sulfur atom of the cysteine residue at the catalytic center of cysteine proteases. In papain family cysteine proteases, the binding of E-64 to the catalytic cysteine, which is located at the bottom of the substrate binding cleft, has been reported to induce only a small structural changes (55–58). This is considered to be the case with Der p 1, and E-64 covers only a small area of the Der p 1 in the model of Der p 1/E-64 complex (Fig. 5*C*). The results indicated that E-64-treated rDer p 1 retained almost all the B cell epitopes, which were reported to be highly conformation dependent (59–61), and the global tertiary structure of rDer p 1, although a

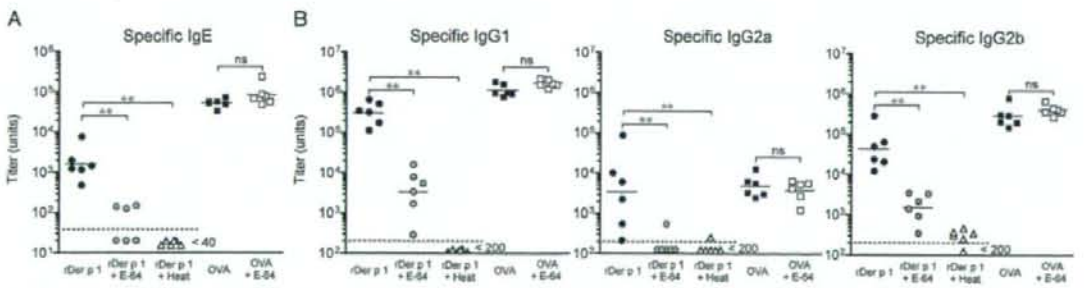

FIGURE 3. Titration for Ag-specific IgE (*A*) and IgGs (*B*) at 4 wk on immunization with rDer p 1 activated with L-cysteine, E-64-treated rDer p 1, heat-denatured Der p 1, OVA, and E-64-treated OVA. The broken lines indicate the smallest dilution factor used, i.e., the highest concentrations of sera in the titration of Der p 1-specific IgE and IgGs are 1/40 and 1/200 dilutions, respectively. Bars indicate geometric means. The Mann-Whitney *U* 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 (**, $p < 0.01$); ns, not significantly different ($p > 0.05$).