IL-4 and IL-13: Their Pathological Roles in Allergic Diseases and their Potential in Developing New Therapies-Update

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Abstract: Bronchial asthma is a complex disease in which a lot of cells and mediators are involved. However, a substantial body of evidence has accumulated pointing to the pivotal role of Th2-cytokines, interleukin (IL)-4, and IL-13, based on expression of these cytokines in the bronchial lesions, genetic association of the signaling molecules of these cytokines, and analyses of mouse models. We previously reviewed the signal transduction of these cytokines, the correlation of these cytokines with the pathogenesis of allergic diseases, and trials to develop reagents targeting these cytokines. Since we published that article, several progresses have been made in this field, so in this article, we summarize the recent topics as for the issues that we picked up before.

Keywords: IL-4, IL-13, IL-4 receptor, IL-13 receptor, allergic disease, bronchial asthma, soluble IL-4 receptor, soluble IL-13 receptor

INTRODUCTION

We reviewed the pathological roles in allergic diseases and the potential in developing new therapies of IL-4 and IL-13 two years ago [1]. We here have added several updated issues related to this.

1. THE MECHANISM OF THE IL-13 RECEPTOR α2 CHAIN (IL-13Rα2) EXPRESSION

Two types of IL-13 receptor exist: the heterodimer composed of the IL-4R α chain (IL-4Rα) and the IL-13 receptor α1 chain (IL-13Rα1), and IL-13Rα2. The hetrodimeric receptor transduces the IL-13 signal intracellularly mainly via the JAK-STAT and the phosphatidylinositol-3 kinase/IRS-1/2 pathways. In contrast, it is confirmed that IL-13Rα2 blocks its signal, acting as a 'decoy receptor' [1-3]. Although the mechanism of IL-13Rα2 expression was obscure, it has recently begun to be clarified.

We showed that in bronchial epithelial cells, only a low amount of IL-13Rα2 is expressed at the constitutive state; however, the stimulation of IL-4 or IL-13 up-regulates expression of IL-13Rα2 [3]. Zheng *et al.* demonstrated that IL-4– or IL-13–transgenic mice show a marked increase of IL-13Rα2 mRNA in lungs, predominantly in airway epithelial cells and macrophages [4]. We furthermore showed IL-13Rα2 expression is also augmented in lungs of asthma-induced mice [3]. In addition to lungs, it was demonstrated by Chiaramonte *et al.* that the burden of the parasitic helminth *Schistoma mansoni*-induces fibrosis in liver, followed by up-regulation of IL-13Rα2 expression [5]. It is confirmed that the promoter activity of the IL-13Rα2 gene is enhanced by IL-4 or IL-13, dependent on STAT6 in the HaCaT human keratinocyte cell line [6]. We and Chiaramonte *et al.* also demonstrated that IL-13Rα2 expression by IL-4 or IL-13 is dependent on STAT6 [3, 5]. These results suggest that induction of IL-4 or IL-13 induces expression of IL-13Rα2 in the STAT6-dependent signal pathway in some cells or tissues and that IL-13Rα2 acts as a negative feedback regulator for the IL-13 signal (Fig. 1).

2. BIOLOGICAL ACTIVITIES OF IL-13 ON NON-IMMUNE CELLS

Although the biological activities of IL-13 on immune cells were well characterized, those on non-immune cells remained unresolved. Because it has been shown that IL-13 is involved in the pathogenesis of bronchial asthma, independent from its actions on immune cells [7], a lot of attention has been paid to understanding biological activities of IL-13 on resident cells in the bronchial tissue. Particularly, the direct actions of IL-13 on bronchial epithelial cells are thought to be of great importance for the pathogenesis of bronchial asthma. The recent report that reconstitution of STAT6 only in bronchial epithelial cells into STAT6-disrupted mice restores IL-13–induced airway hyperresponsiveness (AHR) and mucous production, but not inflammation or fibrosis, supports this notion [8]. The following activities of IL-13 on the resident cells in the bronchial tissues have been reported: (i) production of TGF-β [9, 10] and eotaxin-3 [11], expression of a chloride channel, human CLCA1 (mouse CLCA3) [12], and production of mucin [13] in bronchial epithelial cells; (ii) production of eotaxin [14], expression of integrin [15], and proliferation [16] in fibroblasts; and (iii) production of eotaxin [17, 18] and enhancement of contraction [19-21] in smooth muscle cells.

3. THE EFFECTS OF SOLUBLE IL-4Rα (NUVANCE™) ON BRONCHIAL ASTHMA PATIENTS

As initial trials to apply soluble recombinant IL-4Rα to bronchial asthma patients showed modest benefit, it was hoped that this reagent might be used widely for bronchial

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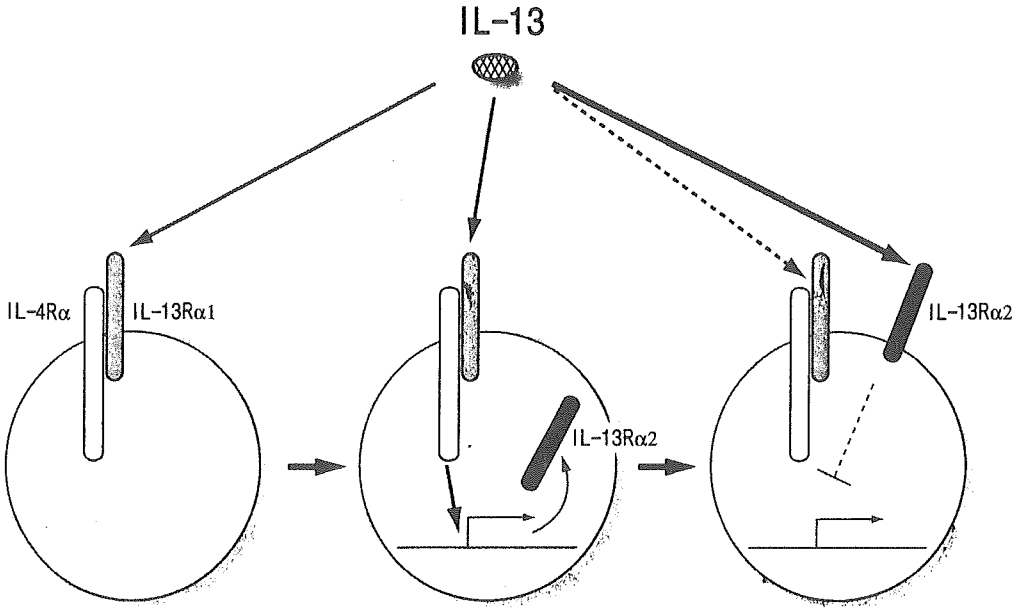

Fig. (1). Negative feedback system of IL-13 signal by IL-13Rα2.
The negative feedback system of IL-13 signal by induction of IL-13Rα2 is depicted. In this system, IL-4 or IL-13 induces expression of IL-13Rα2, which down-regulates IL-13 signal.

asthma patients [22, 23]. However, the Immunex corporation announced in 2001 that further development was discontinued, because the asthma exacerbation rate was not significantly different than in the placebo group (ref.: http://www.clevelandclinicmeded.com/diseasemanagement/pulmonary/asthma/asthma2.htm). It may be because IL-13 has more important role than IL-4 in the pathogenesis of bronchial asthma. Further studies aimed to block the signals of not only IL-4 but also IL-13 would be of interest.

4. THE PATHOPHYSIOLOGICAL ROLE OF SCCA MOLECULES

In order to study potential downstream consequences of IL-13 on human bronchial epithelial cells, we first employed microarrays (HuGeneFL Array, Affymetrix) to identify genes induced by IL-4 or IL-13 in human bronchial epithelial cells [24]. Among the identified genes, two members of the serine proteinase inhibitor (serpin) family of protease inhibitors, SCCA1 (SERPINB3) and SCCA2 (SERPINB4), showed the highest increase. We confirmed SCCA expression by IL-4 or IL-13 at the protein level, with the result that both intracellular expression and secretion of SCCA protein increased in bronchial epithelial cells by IL-4 or IL-13.

Dr. Holgate's group analyzed the expression profiles of cDNA libraries constructed from atopic normal or asthma-derived bronchial biopsies [24]. The number of SCCA-related clones was significantly higher in bronchial tissues of asthma patients than those of normal donors. Furthermore, assessment of serum concentrations of SCCA in asthmatic and non-asthmatic children by ELISA showed that SCCA levels were significantly higher in the asthmatic subjects than in control, non-asthmatic subjects, particularly during the attack phase. These results strongly suggest that SCCA is correlated with the pathogenesis of bronchial asthma, downstream of the IL-4/IL-13 signal pathway.

Although several intrinsic target proteinases for SCCA1 and SCCA2 have been found, the biological roles of SCCA1 and SCCA2 remained obscure. A mite allergen, Der p 1, is one of the most immunodominant allergens, and also acts as a cysteine proteinase probably correlated with the pathogenesis of allergic diseases. We explored the possibility that SCCA proteins target Der p 1, and it turned out that SCCA2, but not SCCA1, inhibited the catalytic activities of Der p 1 [25]. These results suggest that SCCA2 targets an extrinsic cysteine proteinase derived from house dust mites, and that it may have a protective role against mite-causing biological reactions (Fig. 2). We assume that SCCA1 and SCCA2 may protect against extrinsic proteinases derived from not only mite but also other microbes or parasites involved in the defense mechanism of IL-4 and IL-13. In addition to pathophysiological roles of SCCA molecules, it is also hoped that SCCA proteins, or a low-weight compound mimicking SCCA proteins, could be developed as a reagent inhibiting biological reactions of mite allergens, based on our findings.

CONCLUSION

In conclusion, we have described recent advances in signal transduction and biological activities of IL-4 and IL-13 and development of therapeutic reagents targeting these cytokines in this article. We hope that the details of the involvement of these cytokines in the pathogenesis of allergic diseases will be clarified furthermore and that some reagent targeting these cytokines will emerge soon.

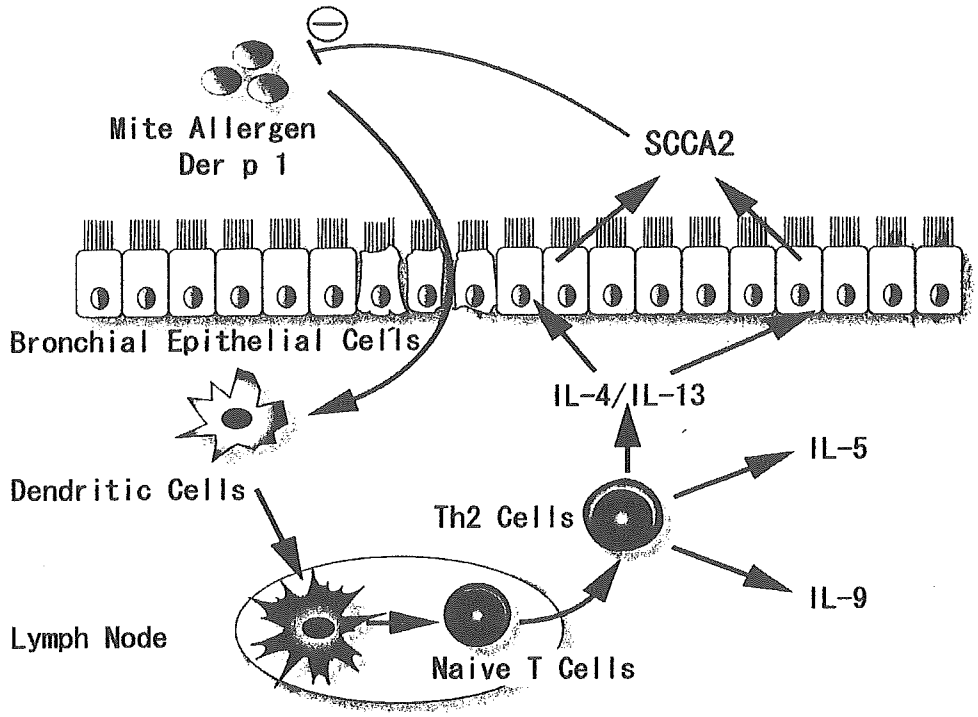

Fig. (2). Protective system by SCCA against a mite allergen, Der p 1.
A possible protective system by SCCA against a mite allergen, Der p 1, is depicted. In this system, IL-4 or IL-13 induces expression of SCCA in bronchial epithelial cells, which would protect against a mite allergen, Der p 1.

ABBREVIATIONS

IL	=	Interleukin
R	=	Receptor
IL-4Rα	=	the IL-4R α chain
γc	=	The IL-2R γ chain
IL-13Rα1	=	The IL-13R α1 chain
PI	=	Phosphatidylinositol
IRS	=	Insulin receptor substrate
ITIM	=	Immunoreceptor tyrosine-based inhibitory motif
SNP	=	Single nuclear polymorphism

REFERENCES

[1] Izuhara, K.; Arima, K.; Yasunaga, S. *Curr. Drug Targets – Inflamm. Allergy*, **2002**, *1*, 263.
[2] Kawakami, K.; Taguchi, J.; Murata, T.; Puri, R.K. *Blood*, **2001**, *97*, 2673.
[3] Bernard, J.; Treton, D.; Vermot-Desroches, C.; Boden, C.; Horellou, P.; Angevin, E.; Galanaud, P.; Wijdenes, J.; Richard, Y. *Lab. Invest.*, **2001**, *81*, 1223.
[4] Yasunaga, S.; Yuyama, N.; Arima, K.; Tanaka, H.; Toda, S.; Maeda, M.; Matsui, K.; Goda, C.; Yang, Q.; Sugita, Y.; Nagai, H.; Izuhara, K. *Cytokine*, **2003**, *24*, 293.
[5] Zheng, T.; Zhu, Z.; Liu, W.; Lee, C.G.; Chen, Q.; Homer, R.J.; Elias, J.A. *J. Allergy Clin. Immunol.*, **2003**, *111*, 720.
[6] Chiaramonte, M.G.; Mentink-Kane, M.; Jacobson, B.A.; Cheever, A.W.; Whitters, M.J.; Goad, M.E.; Wong, A.; Collins, M.; Donaldson, D.D.; Grusby, M.J.; Wynn, T.A. *J. Exp. Med.*, **2003**, *197*, 687.
[7] David, M.D.; Bertoglio, J.; Pierre, J. *Oncogene*, **2003**, *22*, 3386.
[8] Grünig, G.; Warnock, M.; Wakil, A.E.; Venkayya, R.; Brombacher, F.; Rennick, D.M.; Sheppard, D.; Mohrs, M.; Donaldson, D.D.; Locksley, R.M.; Corry, D.B. *Science*, **1998**, *282*, 2261.
[9] Kuperman, D.A.; Huang, X.; Koth, L.L.; Chang, G.H.; Dolganov, G.M.; Zhu, Z.; Elias, J.A.; Sheppard, D.; Erle, D.J. *Nat. Med.*, **2002**, *8*, 885.
[10] Richter, A.; Puddicombe, S.M.; Lordan, J.L.; Bucchieri, F.; Wilson, S.J.; Djukanovic, R.; Dent, G.; Holgate, S.T.; Davies, D.E. *Am. J. Respir. Cell Mol. Biol.*, **2001**, *25*, 385.
[11] Wen, F.Q.; Kohyama, T.; Liu, X.; Zhu, Y.K.; Wang, H.; Kim, H.J.; Kobayashi, T.; Abe, S.; Spurzem, J.R.; Rennard, S.I. *Am. J. Respir. Cell Mol. Biol.*, **2002**, *26*, 484.
[12] Banwell, M.E.; Tolley, N.S.; Williams, T.J.; Mitchell, T.J. *Cytokine*, **2002**, *17*, 317.
[13] Zhou, Y.; Dong, Q.; Louahed, J.; Dragwa, C.; Savio, D.; Huang, M.; Weiss, C.; Tomer, Y.; McLane, M.P.; Nicolaides, N.C.; Levitt, R.C. *Am. J. Respir. Cell Mol. Biol.*, **2001**, *25*, 486.
[14] Zuhdi Alimam, M.; Piazza, F.M.; Selby, D.M.; Letwin, N.; Huang, L.; Rose, M.C. *Am. J. Respir. Cell Mol. Biol.*, **2000**, *22*, 253.
[15] Hoeck, J.; Woisetschläger, M. *J. Immunol.*, **2001**, *166*, 4507.
[16] Doucet, C.; Brouty-Boyé, D.; Pottin-Clemenceau, C.; Jasmin, C.; Canonica, G.W.; Azzarone, B. *Int. Immunol.*, **1998**, *10*, 1421.
[17] Kraft, M.; Lewis, C.; Pham, D.; Chu, H.W. *J. Allergy Clin. Immunol.*, **2001**, *107*, 602.
[18] Moore, P.E.; Church, T.L.; Chism, D.D.; Panettieri, R.A. Jr.; Shore, S.A. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **2002**, *282*, L847.
[19] Hirst, S.J.; Hallsworth, M.P.; Peng, Q.; Lee, T.H. *Am. J. Respir. Crit. Care Med.*, **2002**, *165*, 1161.
[20] Laporte, J.C.; Moore, P.E.; Baraldo, S.; Jouvin, M.H.; Church, T.L.; Schwartzman, I.N.; Panettieri, R.A. Jr.; Kinet, J.P.; Shore, S.A. *Am. J. Respir. Crit. Care Med.*, **2001**, *164*, 141.
[21] Grunstein, M.M.; Hakonarson, H.; Leiter, J.; Chen, M.; Whelan, R.; Grunstein, J.S.; Chuang, S. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **2002**, *282*, L520.
[22] Liu, X.; Kohyama, T.; Wang, H.; Zhu, Y.K.; Wen, F.Q.; Kim, H.J.; Romberger, D.J.; Rennard, S.I. *Am. J. Physiol .Lung Cell Mol. Physiol.*, **2002**, *282*, L1049.

[23] Borish, L.; Nelson, H.; Nathan, B.; Blosch, C. *J. Allergy Clin. Immunol.*, **1998**, *99*, S413.
[24] Borish, L.C.; Nelson, H.S.; Corren, J.; Bensch, G.; Busse, W.W.; Whitmore, J.B.; Agosti, J.M. *J. Allergy Clin. Immunol.*, **2001**, *107*, 963.
[25] Yuyama, N.; Davies, D.E.; Akaiwa, M.; Matsui, K.; Hamasaki, Y.; Suminami, Y.; Lu Yoshida, N.; Maeda, M.; Pandit, A.; Lordan, J.L.; Kamogawa, Y.; Arima, K.; Nagumo, F.; Sugimachi, M.; Berger, A.; Richards, I.; Roberds, S.L.; Yamashita, T.; Kishi, F.; Kato, H.; Arai, K.; Ohshima, K.; Tadano, J.; Hamasaki, N.; Miyatake, S.; Sugita, Y.; Holgate, S.T.; Izuhara, K. *Cytokine*, **2002**, *19*, 287.
[26] Sakata, Y.; Arima, K.; Takai, T.; Sakurai, W.; Masumoto, K.; Yuyama, N.; Suminami, Y.; Kishi, F.; Yamashita, T.; Kato, T.; Ogawa, H.; Fujimoto, K.; Matsuo, Y.; Sugita, Y.; Izuhara, K. *J. Biol. Chem.*, **2004**, *279*, 5081.

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Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity

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Abstract

Although mite major group 1 allergens, Der p 1 and Der f 1, were first isolated as cysteine proteases, some studies reported that natural Der p 1 exhibits mixed cysteine and serine protease activity. Clarifying whether the serine protease activity originates from Der p 1 or is due to contamination is important for distinguishing between the pathogenic proteolytic activities of group 1 allergens and mite-derived serine proteases. Recombinant mite group 1 allergens would be useful tool for addressing this issue, because they are completely free from contamination by mite serine proteases. Recombinant Der p 1 and Der f 1, and highly purified natural forms exhibited only cysteine protease activity. However, commercially available natural forms exhibited both activities, but the two activities were eluted into different fractions in size-exclusion column chromatography. The substrate specificity associated with the serine protease activity was similar to that of Der f 3. These results indicate that the serine protease activity does not originate from group 1 allergens.

Keywords: Recombinant major house dust mite group 1 allergens; Der p 1; Der f 1; Cysteine protease; Group 3 allergens; Serine protease

House dust mites of the *Dermatophagoides* species (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are associated with various allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis, and are the most important causative factor of in-door allergens [1–3]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens are considered to be the major allergens derived from house dust mites based on the frequency of patients sensitized by them, amount of specific IgE produced against them, and their content in mite extract [4]. Group 1 allergens exist in abundance in mite fecal pellets and account for over 50% of the IgE antibodies produced against total mite extract. Der p 1 of *D. pteronyssinus* and Der f 1 of *D. farinae* have 82% sequence homology in the amino acid sequences of their mature forms, which corresponds to their natural allergen state, and the sequences are homologous to those of papain-like cysteine proteases [5,6]. Recently, the cysteine protease activity of Der p 1 was suggested to be involved in the pathogenesis of allergy [7–15].

Although Der p 1 and Der f 1 were first isolated as cysteine proteases, some studies reported that natural Der p 1 exhibits mixed cysteine and serine protease activity [11,16,17]. The specificity for substrates and

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inhibitors associated with such serine protease activity within their preparations was different from that of the cysteine protease activity. However, house dust mites produce at least three groups of serine proteases, group 3, 6, and 9 allergens [18–21], and therefore, the possibility of contamination of cysteine protease Der p 1 preparations with these serine proteases cannot be excluded. Clarifying whether the serine protease activity originates from Der p 1 or is due to contamination is important for distinguishing between the pathogenic proteolytic activities of mite major group 1 allergens and mite-derived serine proteases. Recombinant mite group 1 allergens as recently reported [22–24] would be useful tool for addressing this issue, because they are completely free from contamination by mite serine proteases.

Using synthetic substrates and irreversible class-specific inhibitors, we compared the specificity of four types of preparation of group 1 allergens: recombinant Der p 1 and Der f 1 expressed in yeast *Pichia pastoris* with yeast-derived N-glycosylation, those without N-glycosylation, commercially available natural types, and natural types purified using a different method. Consequently, we demonstrate that the serine protease activity does not originate from group 1 allergens and that the substrate specificity of the contaminated serine protease is similar to that of a mite group 3 allergen Der f 3.

Materials and methods

Natural Der p 1 and Der f 1. Commercially available natural Der p 1 and Der f 1 were purchased from Indoor Biotechnologies (Charlottesville, VA, USA) and Asahi Breweries (Tokyo, Japan), respectively. Purification of natural Der p 1 and Der f 1 from whole culture extracts of house dust mites by the method of Yasueda et al. [25] was performed. Protein concentration was determined by the Bradford procedure using a protein assay kit (Bio-Rad, Richmond, CA, USA) with bovine IgG (Bio-Rad) as the standard.

Recombinant Der p 1 and Der f 1. Recombinant Der p 1 and Der f 1 were prepared as previously described with some modifications [22–24]. Briefly, proforms of four recombinant types, Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT, were secreted into the culture supernatant of yeast *P. pastoris* and converted into their prosequence-removed mature forms. The mature forms were then purified by anion exchange column and size-exclusion column chromatography.

Measurement of proteolytic activity. Cysteine protease activity was measured as previously described with modifications [23,26]. Enzymes (400 nM) were incubated with or without 1 mM dithiothreitol (DTT) for 5 min at 37 °C in 25 μl. After further incubation with or without E-64 (50 μM) (Peptide Institute, Osaka, Japan) and/or 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (500 μM) (Sigma, St. Louis, MO, USA) for 15 min in 50 μl, assays were conducted in reaction buffer with substrates (0.1 mM) at 37 °C in 100 μl scale. The final concentrations of the enzymes were 100 nM, and those of the inhibitors were 25 μM for E-64 and 250 μM for AEBSF. The substrates used were: butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA), succinyl-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), succinyl-Ala-Ala-Ala-MCA (Suc-AAA-MCA), butyloxycarbonyl-Phe-Ser-Arg-MCA (Boc-FSR-MCA), butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-QGR-MCA), butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA), benzoyl-Arg-MCA (Bz-R-MCA), and succinyl-Ala-Ala-Pro-Phe-MCA (Suc-AAPF-MCA) (Peptide Institute). The fluorescence of aminomethylcoumarin released from the substrate was measured.

Protein sequencing. Samples of commercial natural Der p 1 and Der f 1 before and after SDS–PAGE and electroblotting onto membranes were subjected to N-terminal amino acid sequencing using protein sequencers [23,27].

Size-exclusion column chromatography. Recombinant and natural Der p 1 and Der f 1 were subjected to size-exclusion column chromatography on Protein-Pak 125 (Waters, Milford, MA, USA), and the absorbance at 280 nm was monitored. The eluted fractions were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and silver stained (Silver stain II kit wako; Wako, Osaka, Japan).

Results

Substrate specificity of recombinant and commercial natural Der p 1 and Der f 1

Measurement of the proteolytic activity using eight synthetic substrates revealed that the activity of recombinant mite group 1 allergens with yeast-derived hyperglycosylation (Der p 1-WT and Der f 1-WT) and that of commercial natural types were different from each other in terms of substrate specificity and DTT-dependence (Fig. 1). The patterns of substrate specificity and DTT-dependence were similar between Der p 1-WT and Der f 1-WT, and also between commercial Der p 1 and Der f 1.

Der p 1-WT and Der f 1-WT showed DTT-dependent proteolytic activity against six synthetic substrates (Figs. 1A–F) and almost no activity against two (Figs. 1G and H). This DTT-dependency suggested that the activity of Der p 1-WT and Der f 1-WT was that corresponding to a cysteine protease. On the other hand, the commercial natural types showed proteolytic activity and high DTT-dependence against three substrates (Figs. 1A–C); that with low or no DTT-dependence against three substrates (Figs. 1D–F), and little or no activity against two substrates (Figs. 1G and H). This suggested that commercial natural Der p 1 and Der f 1 possessed the other class of proteases as well as that of cysteine protease activity.

Activity of recombinant and natural Der p 1 and Der f 1 treated with class-specific irreversible protease inhibitors

To identify the class of proteases exhibiting DTT-independent activity among the commercial natural allergens (Figs. 1D–F, right two panels) and to reveal whether the difference between recombinant and commercial natural allergens was caused by the hyperglycosylation of recombinant allergens and/or by the method used for purification, we analyzed recombinant allergens without glycosylation (Der p 1-N52Q and Der f 1-N53Q) and natural allergens purified by the method

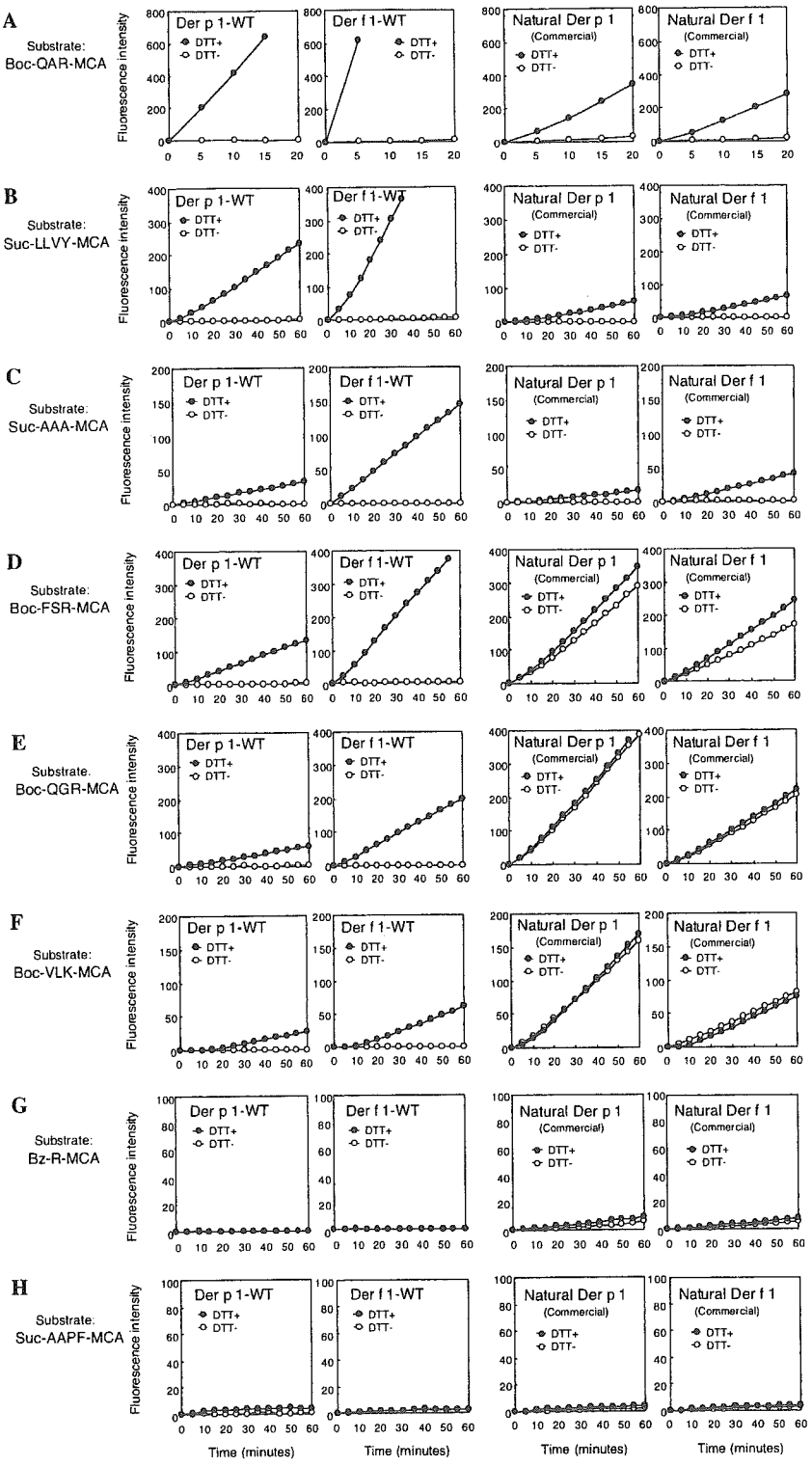


Fig. 1. Hydrolysis of synthetic substrates by recombinant and commercial natural Der p 1 and Der f 1. Eight substrates (A–H) were used. WT: recombinant forms with N-glycosylation. Commercial: commercial natural allergens. The reaction was performed in the presence (DTT+) or absence of DTT (DTT−).

by Yasueda et al. [25] and compared them with Der p 1-WT, Der f 1-WT, and commercial natural allergens using two irreversible class-specific protease inhibitors, cysteine protease-specific E-64 and serine protease-specific AEBSF (Figs. 2 and 3). We selected Boc-QAR-MCA and Boc-QGR-MCA for the substrates, because they are typical substrates suitable for measuring DTT-dependent and -independent activities of commer-

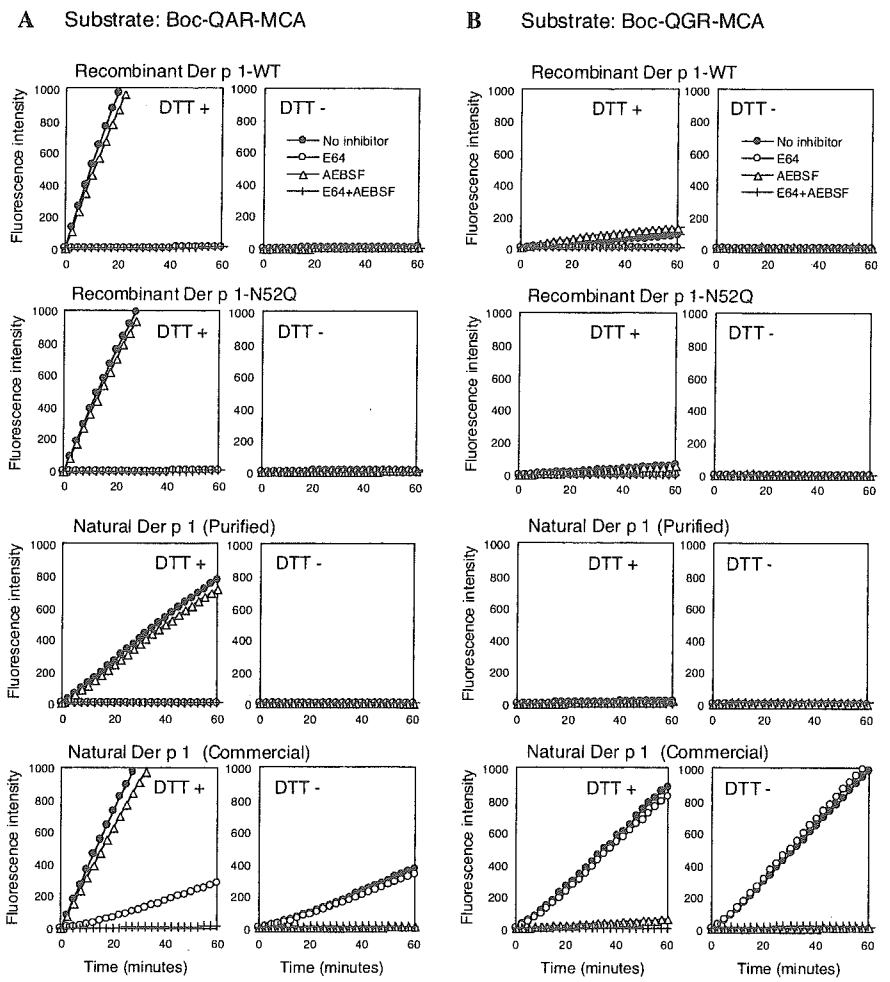


Fig. 2. Activity of recombinant and natural Der p 1 treated with irreversible class-specific protease inhibitors. Boc-QAR-MCA (A) and Boc-QGR-MCA (B) were used as substrates. Der p 1-WT: recombinant Der p 1 with yeast-derived N-glycosylation. Der p 1-N52Q: recombinant Der p 1 without N-glycosylation. Der p 1 (purified): natural Der p 1 purified by the method of Yasueda et al. [25]. Der p 1 (commercial): commercial natural Der p 1. The reaction was performed in the absence of inhibitors or in the presence of E-64, AEBSF or E-64 and AEBSF; and in the presence (DTT+) or absence of DTT (DTT−).

cial natural allergens, respectively (Figs. 1A and E, right two panels).

The activities of recombinant allergens and natural types purified by the method of Yasueda et al. against Boc-QAR-MCA (Figs. 2A and 3A, upper six panels) and Boc-QGR-MCA (Figs. 2B and 3B, upper six panels) were DTT-dependent, and E-64 completely inhibited the activities while AEBSF did not. This indicated that recombinant allergens and natural allergens purified by this method only possessed cysteine protease activity and that hyperglycosylation of recombinant allergens did not affect the activity.

On the other hand, the activities of commercial natural allergens against Boc-QAR-MCA consisted of major DTT-dependent and minor DTT-independent activities (Figs. 2A and 3A, panels at the bottom), and those against Boc-QGR-MCA possessed inverse major DTT-independent and minor DTT-dependent activities (Figs. 2B and 3B, panels at the bottom). The DTT-dependent and -independent activities of commercial natural allergens were inhibited by E-64 and AEBSF, respectively, and the addition of E-64 and AEBSF was necessary for complete inhibition in the presence of DTT. This indicated that the activity of commercial natural allergens consisted of cysteine protease and serine protease activity.

Substrate specificity of cysteine protease activity of recombinant and commercial natural Der p 1 and Der f 1

To compare the substrate specificity of the cysteine protease associated activities of recombinant and commercial natural allergens, DTT-dependent activities were calculated from the results shown in Fig. 1. The patterns of relative DTT-dependent activities were similar between recombinant and commercial natural allergens (Fig. 4A), although absolute DTT-dependent activity was higher for recombinant allergens than for commercial natural allergens (Fig. 1). This indicated

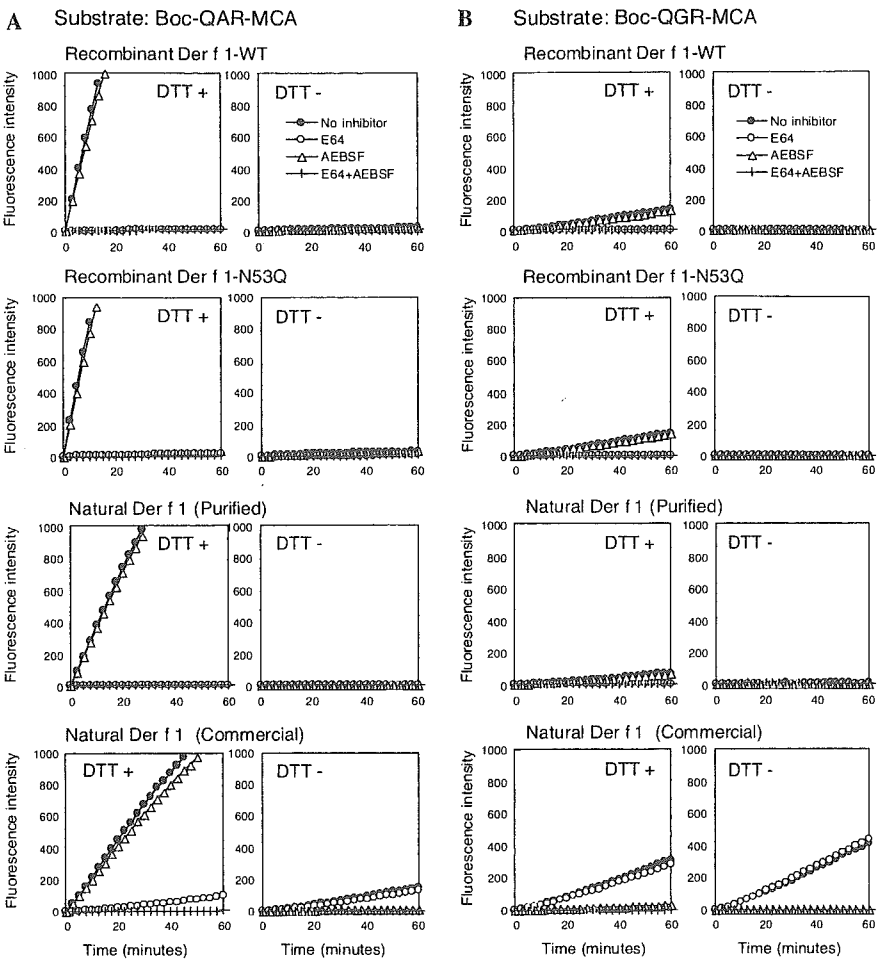


Fig. 3. Activity of recombinant and natural Der f 1 treated with class-specific irreversible protease inhibitors. The reaction was performed as that in Fig. 2, except that recombinant and natural Der f 1 were used.

that the substrate specificity of the cysteine protease associated activities was similar between recombinant and commercial natural allergens.

Substrate specificity of serine protease associated activity of commercial natural Der p 1 and Der f 1

The substrate specificity of commercial natural allergens with DTT-independent activity (Fig. 1) was compared with the previously described specificity of mite serine proteases, except group 9 allergens, whose substrate specificity was not well characterized, and was found to be similar to the mite group 3 allergen Der f 3 but not to group 6 allergens (Fig. 4B, Boc-FSR-MCA, Boc-VLK-MCA, Suc-AAPF-MCA, and Suc-LLVY-MCA).

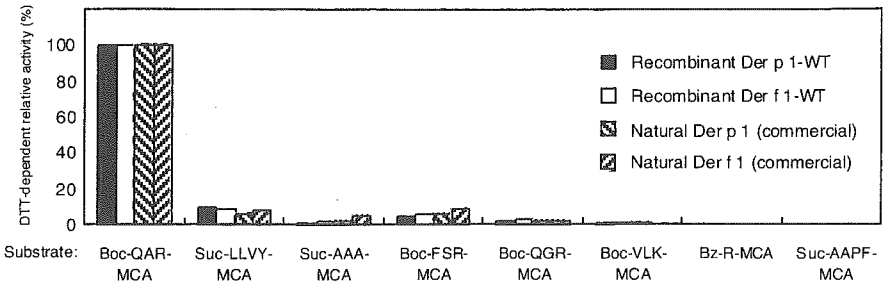
N-terminal sequencing of commercial natural Der p 1 and Der f 1

To determine the content of the serine protease within the commercial natural allergens, they were subjected to protein sequencing. N-terminal sequences for Der p 1 and Der f 1 were detected, but those for mite serine proteases that belong to three groups, group 3, 6, and 9 allergens, were not detectable. This indicated that contamination of mite serine proteases was minute if the serine protease activity was mite-derived. However, it was possible that isoforms of Der p 1 and Der f 1 exhibited the serine protease activity [16]. Therefore, to determine whether the cysteine and serine protease activities of the commercial natural allergens were from identical molecular species or not, we next analyzed the commercial natural allergens using size-exclusion column chromatography.

Size-exclusion column chromatography of recombinant and natural Der p 1 and Der f 1

The peak activity against Boc-QAR-MCA, which was used to detect cysteine protease activity in the presence of DTT, and that against Boc-QGR-MCA to detect serine protease activity in the absence of DTT were eluted into different fractions (Fig. 5, panels at

A



B

Substrate	DTT-independent relative activity (%)				
	Der p 1 (DTT -) (commercial)	Der f 1 (DTT -) (commercial)	Der f 3	Der p 6	Der f 6
Boc-QGR-MCA	135	120	n.d.	n.d.	n.d.
Boc-FSR-MCA	100	100	100	0.7	2
Boc-VLK-MCA	55	47	58	0.2	1.2
Suc-AAPF-MCA	0.9	1.8	0	100	100
Suc-LLVY-MCA	0.2	0.7	0	52	50
Suc-AAA-MCA	0	0.6	n.d.	n.d.	n.d.
Boc-QAR-MCA	46	45	n.d.	n.d.	n.d.
Bz-R-MCA	2.2	3.5	n.d.	n.d.	n.d.

n.d.: not determined

Fig. 4. Substrate specificity associated with the protease activity of recombinant and commercial natural Der p 1 and Der f 1. (A) Comparison of the specificity of cysteine protease activity. The activity for each substrate is shown as the relative DTT-dependent fluorescence intensity to that for the substrate Boc-QAR-MCA. The DTT-dependent fluorescence intensity used was calculated by subtracting the intensity in the absence of DTT from that in the presence of DTT. (B) Comparison of the specificity of serine protease activity. The activity for each substrate is shown as the relative DTT-independent fluorescence intensity to that for the substrate Boc-FSR-MCA in commercial natural Der p 1 and Der f 1, and natural Der f 3; and for Boc-AAPF-MCA in natural Der p 6 and Der f 6. The DTT-independent fluorescence intensity used was the intensity in the absence of DTT. The values for natural Der f 3, Der p 6, and Der f 6 were obtained from a previous report [20].

the bottom). The peaks for the cysteine protease activity of Der p 1-N52Q, Der f 1-N53Q, and the natural types purified by the method of Yasueda et al. were eluted into a fraction at an elution time equivalent to that associated with the cysteine protease activity of the commercial natural allergens (Fig. 5, middle four panels). Those for Der p 1-WT and Der f 1-WT were eluted earlier reflecting their larger molecular sizes (Fig. 5, panels at the top). The shapes of protein–elution curves and band density on SDS–PAGE of the eluted fractions were correlated well with the strength of the cysteine protease activity [22] but not with that of the serine protease activity (data not shown). These results indicated that the cysteine and serine protease activities associated with the commercial natural allergens originated from different molecular species and that the serine protease had an apparent molecular size different from those of Der p 1 and Der f 1.

Discussion

Several studies have found that cysteine and serine protease activities are associated with affinity-purified natural Der p 1 [11,16,17], and we obtained similar results using commercial natural Der p 1 and Der f 1. However, recombinant Der p 1 and Der f 1, and natural allergens purified by the method of Yasueda et al. only exhibited cysteine protease activity (Figs. 1–3). We showed that the substrate specificities of commercial natural Der p 1 and Der f 1 are similar to those of recombinant forms for DTT-dependent cysteine protease activity (Fig. 4A), and similar to that of a mite group 3 allergen Der f 3 for DTT-independent serine protease activity (Fig. 4B). The cysteine and serine protease activities eluted into different fractions during size-exclusion column chromatography (Fig. 5), indicating that these different types of protease activity are due to different molecular species. These results indicate that the major house dust mite group 1 allergens have only cysteine protease activity and that the serine protease activity detected within some preparations of natural group 1 allergens is due to contamination by serine protease with similar substrate specificity to Der f 3.

Hewitt et al. [11] reported that natural Der p 1, which was affinity-purified using a column coupled with murine anti-Der p 1 monoclonal antibody (mAb), exhibited cysteine and serine protease activities even after further active-site affinity purification using a thiol column. They showed that different substrate specificities associated with the cysteine and serine proteases are present using insulin B chain as a model peptide [16]. Recently, Brown et al. [17] reported similar results using two synthetic substrates, Boc-QAR-MCA and Tosyl-Gly-Pro-Arg-MCA;

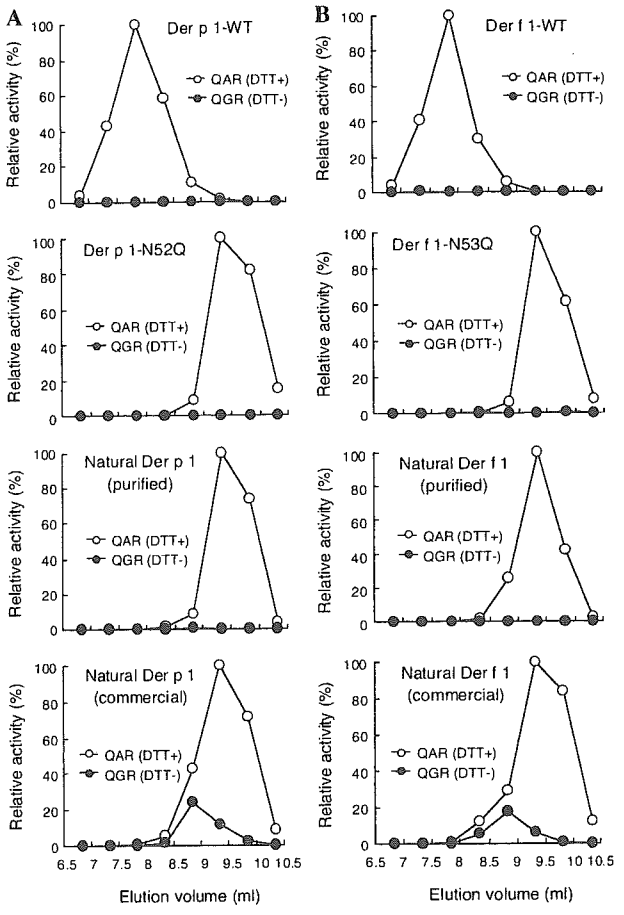


Fig. 5. Proteolytic activity associated with eluted fractions of recombinant and natural Der p 1 and Der f 1 separated by size-exclusion column chromatography. (A) Der p 1. (B) Der f 1. Eluted fractions were subjected to the measurement of proteolytic activity against the substrate Boc-QAR-MCA in the presence of DTT [QAR (DTT+)] and against Boc-QGR-MCA in the absence of DTT [QGR (DTT−)]. The relative fluorescence intensity to the peak intensity for Boc-QAR-MCA is shown.

and in the present study, we too observed similar behavior using several synthetic substrates of commercial natural Der p 1 and Der f 1, although the strength of the serine protease activity varied by lot and manufacturers (data not shown). Schulz et al. [26] and Brown et al. [17] reported that the serine protease found within the preparation of affinity-purified natural Der p 1 could be removed by passing it through a column coupled with soybean trypsin inhibitor (SBTI). However, whether such serine protease activity is associated with the group 1 allergens or contamination by mite-derived serine proteases [18–21] has not been clarified. We showed that recombinant Der p 1 and De f 1 purified by a simple method [22,23] and natural allergens purified by another method which used many purification steps without mAb and SBTI [25] exhibited only cysteine protease activity. Furthermore, we indicated that the serine proteases within commercial Der p 1 and Der f 1 have molecular sizes different from that of the cysteine protease and that their substrate specificities are similar to that of Der f 3 but not to those of Der p 6 and Der f 6 [20]. Therefore, the identity of the serine protease activity might be the group 3 allergens. The larger molecular sizes of the peaks associated with serine protease activity for commercial group 1 allergens compared to those of the peaks associated with cysteine protease activity for size-exclusion column chromatography do not conflict with the higher molecular weights of group 3 allergens Der p 3 and Der f 3 compared to those of the group 1 allergens estimated by SDS–PAGE [19,28].

Co-purification of serine protease activity with cysteine protease Der p 1 by both affinity purification using the mAb-coupled column and active-site affinity purification was reported by Hewitt et al. [11]. Possibilities that the residual serine protease activity is due to insufficient column washing after loading of the extract to the columns or that the serine protease have weak affinity for column materials or the murine mAb used are not to be excluded, as we have observed that some preparations of whole culture extracts of mites showed a much higher proteolytic activity for serine protease activity than cysteine protease activity. Der p 3 might bind to the thiol column via Cys12, which is predicted to be an unpaired cysteine residue based on its homology with trypsin proteins from various species [29].

The cysteine protease activity of Der p 1 is suggested to be involved in the pathogenesis of allergy [7–15]. Mite-derived serine protease activity also has biological activities including the ability to disrupt tight junctions [30], stimulate bronchial epithelial cells by cleaving the protease activated receptor-2 (PAR-2) [31], cleave an intrinsic protease inhibitor, elafin [17], and produce anaphylatoxins by cleaving complement components [32]. Although we considered that the amount of the serine proteases that contaminated the commercial group 1 allergens was small because no peaks associated with the group 3, 6, and 9 allergens were detected by protein sequencing analysis, their activities were not negligible. Therefore, recombinant Der p 1 and Der f 1, which can be easily prepared and exhibit only cysteine protease activity, will be useful for the accurate analysis of the pathogenicity of the cysteine protease activity of the major mite group 1 allergens, which are distinct from those of mite serine proteases. Additionally, further analysis of the contribution of serine protease activity including that by the group 3 allergens, which could have significant activities and different substrate specificities from the major group 1 allergens [17], to the pathogenesis of allergy may also be important.

Acknowledgments

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References

[1] R. Voorhorst, F.T.M. Spieksma, M.J. Vanekamp, M.J. Leupen, A.W. Lyklema, The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces. Identity with the house dust allergen, J. Allergy 39 (1967) 325–328.

[2] T. Miyamoto, S. Oshima, T. Ishizaki, S.H. Sato, Allergenic identity between the common floor mite (*Dermatophagoides farinae* Hughes, 1961) and house dust as a causative antigen in bronchial asthma, J. Allergy 42 (1968) 14–28.

[3] T.A. Platts-Mills, M.D. Chapman, Dust mites: Immunology, allergic disease, and environmental control, J. Allergy Clin. Immunol. 80 (1987) 755–775.

[4] W.R. Thomas, W.A. Smith, B.J. Hales, K.L. Mills, R.M. O'Brien, Characterization and immunobiology of house dust mite allergens, Int. Arch. Allergy Immunol. 129 (2002) 1–18.

[5] K.Y. Chua, G.A. Stewart, W.R. Thomas, R.J. Simpson, R.J. Dilworth, T.M. Plozza, K.J. Turner, Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p* 1. Homology with cysteine proteases, J. Exp. Med. 167 (1988) 175–182.

[6] R.J. Dilworth, K.Y. Chua, W.R. Thomas, Sequence analysis of cDNA coding for a major house dust mite allergen, *Der f* I, Clin. Exp. Allergy 21 (1991) 25–32.

[7] F. Shakib, O. Schulz, H. Sewell, A mite subversive: Cleavage of CD23 and CD25 by Der p 1 enhances allergenicity, Immunol. Today 19 (1998) 313–316.

[8] L. Gough, O. Schulz, H.F. Sewell, F. Shakib, The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response, J. Exp. Med. 190 (1999) 1897–1902.

[9] E.E. Comoy, J. Pestel, C. Duez, G.A. Stewart, C. Vendeville, C. Fournier, F. Finkelman, A. Capron, G. Thyphronitis, The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN-γ, J. Immunol. 160 (1998) 2456–2462.

[10] H. Wan, H.L. Winton, C. Soeller, E.R. Tovey, D.C. Gruenert, P.J. Thompson, G.A. Stewart, G.W. Taylor, D.R. Garrod, M.B. Cannell, C. Robinson, Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions, J. Clin. Invest. 104 (1999) 123–133.

[11] C.R. Hewitt, A.P. Brown, B.J. Hart, D.I. Pritchard, A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: Innate protection by antiproteases, J. Exp. Med. 182 (1995) 1537–1544.

[12] Y. Sakata, K. Arima, T. Takai, W. Sakurai, K. Masumoto, N. Yuyama, Y. Suminami, F. Kishi, T. Yamashita, T. Kato, H. Ogawa, K. Fujimoto, Y. Matsuo, Y. Sugita, K. Izuhara, The squamous cell carcinoma antigen 2 inhibits the cysteine proteinase activity of a major mite allergen, Der p 1, J. Biol. Chem. 279 (2004) 5081–5087.

[13] O. Schulz, H.F. Sewell, F. Shakib, Proteolytic cleavage of CD25, the α subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity, J. Exp. Med. 187 (1998) 271–275.

[14] A.M. Ghaemmaghami, L. Gough, H.F. Sewell, F. Shakib, The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: Allergen-induced Th2 bias determined at the dendritic cell level, Clin. Exp. Allergy 32 (2002) 1468–1475.

[15] N. Asokananthan, P.T. Graham, D.J. Stewart, A.J. Bakker, K.A. Eidne, P.J. Thompson, G.A. Stewart, House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: The cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1, J. Immunol. 169 (2002) 4572–4578.

[16] C.R. Hewitt, H. Horton, R.M. Jones, D.I. Pritchard, Heterogeneous proteolytic specificity and activity of the house dust mite proteinase allergen Der p I, Clin. Exp. Allergy 27 (1997) 201–207.

[17] A. Brown, K. Farmer, L. MacDonald, N. Kalsheker, D. Pritchard, C. Haslett, J. Lamb, J.M. Sallenave, House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors, Am. J. Respir. Cell. Mol. Biol. 29 (2003) 381–389.

[18] G.A. Stewart, M.R. Kollinger, C.M. King, P.J. Thompson, A comparative study of three serine proteases from *Dermatophagoides pteronyssinus* and *D. farinae*, Allergy 49 (1994) 553–560.

[19] G.A. Stewart, L.D. Ward, R.J. Simpson, P.J. Thompson, The group III allergen from the house dust mite *Dermatophagoides pteronyssinus* is a trypsin-like enzyme, Immunology 75 (1992) 29–35.

[20] H. Yasueda, H. Mita, K. Akiyama, T. Shida, T. Ando, S. Sugiyama, H. Yamakawa, Allergens from *Dermatophagoides* mites with chymotryptic activity, Clin. Exp. Allergy 23 (1993) 384–390.

[21] C. King, R.J. Simpson, R.L. Moritz, G.E. Reed, P.J. Thompson, G.A. Stewart, The isolation and characterization of a novel collagenolytic serine protease allergen (Der p 9) from the dust mite *Dermatophagoides pteronyssinus*, J. Allergy Clin. Immunol. 98 (1996) 739–747.

[22] T. Takai, T. Kato, H. Yasueda, K. Okumura, H. Ogawa, Analysis of the structure and allergenicity of recombinant pro- and mature Der p 1 and Der f 1: Major conformational IgE-epitopes blocked by prodomains, J. Allergy Clin. Immunol. doi:10.1016/j.jaci.2004.11.024.

[23] T. Takai, R. Mineki, T. Nakazawa, M. Takaoka, H. Yasueda, K. Murayama, K. Okumura, H. Ogawa, Maturation of the activities of recombinant mite allergens Der p 1 and Der f 1, and its implication in the blockade of proteolytic activity, FEBS Lett. 531 (2002) 265–272.

[24] T. Yasuhara, T. Takai, T. Yuuki, H. Okudaira, Y. Okumura, Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding, Clin. Exp. Allergy 31 (2001) 116–124.

[25] H. Yasueda, H. Mita, Y. Yui, T. Shida, Isolation and characterization of two allergens from *Dermatophagoides farinae*, Int. Arch. Allergy Appl. Immunol. 81 (1986) 214–223.

[26] O. Schulz, H.F. Sewell, F. Shakib, A sensitive fluorescent assay for measuring the cysteine protease activity of Der p 1, a major allergen from the dust mite *Dermatophagoides pteronyssinus*, Mol. Pathol. 51 (1998) 222–224.

[27] R. Mineki, H. Taka, T. Fujimura, M. Kikkawa, N. Shindo, K. Murayama, In situ alkylation with acrylamide for identification of cysteinyl residues in proteins during one- and two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis, Proteomics 2 (2002) 1672–1681.

[28] T. Ando, R. Homma, Y. Ino, G. Ito, A. Miyahara, T. Yanagihara, H. Kimura, S. Ikeda, H. Yamakawa, M. Iwaki, Y. Okumura, M. Suko, M. Haida, H. Okudaira, Trypsin-like protease of mites: Purification and characterization of trypsin-like protease from mite faecal extract *Dermatophagoides farinae*. Relationship between trypsin-like protease and Der f III, Clin. Exp. Allergy 23 (1993) 777–784.

[29] W.A. Smith, K.Y. Chua, M.C. Kuo, B.L. Rogers, W.R. Thomas, Cloning and sequencing of the *Dermatophagoides pteronyssinus*

group III allergen, Der p III, Clin. Exp. Allergy 24 (1994) 220–228.

[30] H. Wan, H.L. Winton, C. Soeller, G.W. Taylor, D.C. Gruenert, P.J. Thompson, M.B. Cannell, G.A. Stewart, D.R. Garrod, C. Robinson, The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*, Clin. Exp. Allergy 31 (2001) 279–294.

[31] G. Sun, M.A. Stacey, M. Schmidt, L. Mori, S. Mattoli, Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells, J. Immunol. 167 (2001) 1014–1021.

[32] K. Maruo, T. Akaike, T. Ono, T. Okamoto, H. Maeda, Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease, J. Allergy Clin. Immunol. 100 (1997) 253–260.

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Relationship between IL-1β gene polymorphism and gastric mucosal IL-1β levels in patients with *Helicobacter pylori* infection

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Background. Interkeukin-1 (IL-1) gene cluster polymorphisms that are thought to enhance the production of IL-1β are associated with an increased risk of gastric cancer. To determine the role of host genetic factors in *Helicobacter pylori* infection, we examined the relationship between gastric mucosal IL-1β levels and *IL-1B* polymorphisms in patients with *H. pylori* infection. ***Methods.*** Biopsy tissues obtained from 99 patients were homogenized and gastric mucosal IL-1β levels were measured by enzyme-linked immunosorbent assay (ELISA). Single-base polymorphisms at positions −511 and −31 in *IL-1B* were analyzed. ***Results.*** The IL-1β level in the antrum was significantly higher in genotype *IL-1B*-511C/C than in *H. pylori*-negative patients ($P < 0.05$). The *IL-1B* polymorphism did not influence the degree of gastric neutrophil and mononuclear cell infiltration, or gastric atrophy. IL-1β levels in the corpus, but not those in the antrum, correlated to the severity of gastric atrophy. ***Conclusions.*** These findings indicate that *IL-1B* polymorphisms enhance IL-1β production in the antrum; however, other factors might regulate the production of IL-1β in the corpus of the stomach, regardless of *IL-1B* polymorphisms, and high IL-1β production may be associated with the grade of gastric atrophy in the corpus mucosa in patients with *H. pylori* infection.

Key words: *H. pylori* infection, interleukin-1B gene polymorphisms, interleukin-1β

Introduction

Helicobacter pylori is a spiral bacterium that colonizes the human stomach.[1] *H. pylori* plays a critical role in the pathogenesis of peptic ulcers and in the chronic inflammatory process, which may be linked to gastric cancer and gastric lymphoma.[2,3] However, why some patients with *H. pylori* infection have only mild asymptomatic gastritis, and why some develop peptic ulcers and gastric cancer, remains unknown.

The key pathophysiological event in *H. pylori* infection is the initiation of an inflammatory response. Cytokines have been suggested to mediate the mucosal inflammation caused by *H. pylori*.[4] interleukin (IL)-1β is a potent proinflammatory cytokine and is upregulated in the presence of *H. pylori*.[5,6] IL-1β is also a potent inhibitor of gastric acid secretion.[7] The genes for IL-1α and IL-1β, and the IL-1 receptor antagonist gene (*IL-1RN*) are all located on the long arm of chromosome 2.[8,9] Three diallelic polymorphisms in *IL-1B* have been reported, all representing C-to-T base transitions at positions −511, −31, and +3954.[10] A penta-allelic polymorphism, consisting of a variable number of an 86-base pair identical tandem repeat (VNTR), has also been reported. An increase in both the frequency and carriage rate of the less common allele 2 of *IL-1RN* has been associated with increased frequency of several inflammatory diseases.[11] Recently, polymorphisms of the *IL-1B* and *IL-1RN* genes were reported to be associated with gastric cancer risk.[12]

To determine the role of host genetic factors in *H. pylori* infection, we examined the relationship between gastric mucosal IL-1β levels and *IL-1B* polymorphisms in patients with *H. pylori* infection.

Patients and methods

Subjects

Ninety-nine patients (M/F, 56/43; age range, 22–80 years; mean, 50 years) were examined. The study was approved by the Tokai University Hospital Ethics

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Committee, and informed consent was obtained from all patients. Six biopsy specimens were obtained. *H. pylori* infection was diagnosed by histology and cultivation. Biopsy tissue obtained from the antrum and body of the stomach were homogenized separately, and mucosal IL-1β levels were then measured by enzyme-linked immunosorbent assay (ELISA).

Determination of gene polymorphism

Peripheral blood samples were obtained from the 99 patients, and genomic DNA was then extracted, using a DNA extraction kit (Takara, Otsu, Japan).

Polymorphism

Single-base polymorphisms at positions −511 and −31 in the promoter region of *IL-1B* were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The oligonucleotide primers for the analysis of base −511 were 5′-TGGCATTGAT CTGGTTCATC-3′ and 5′-GTTTAGGAATCTTTCC CACTT, while the primer pair for base −31 was 5′-AGCTTCCACCAATACTCTTTTCCCCTTTCC-3′ and 5′-TACACACAAAGAGGCAGAGAGACAGA G-3. The PCR conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 1 min, followed by 72°C for 1 min, and finally 72°C for 7 min. The PCR products were digested at 37°C overnight, with *AvaI* (Takara) for analysis of base −511 and with *AluI* for base −31. The products were then separated by 3% agarose gel electrophoresis and stained with ethidium bromide.

Gastric mucosal IL-1β levels

Both antrum and corpus biopsy tissues were homogenized in 1 ml of phosphate-buffered saline (pH 7.4), using a homogenizer, and then centrifuged at 1800 rpm for 10 min. The supernatants were kept at −20°C until the assay. The IL-1β levels in the biopsy supernatants were determined using a sandwich-type IL-1β ELISA kit (R&D Systems, Minneapolis, MN, USA). Protein content was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Results were expressed as picograms per milligram protein.

Histological evaluation

The biopsy specimens from the antrum and corpus were fixed in 10% buffered formalin. The extent of *H. pylori* infection, neutrophil infiltration, mononuclear cell infiltration, and atrophy were assessed according to the Updated Sydney System and scored from 0 to 3.[13] A pathologist assessed histological findings, and was blinded to the information of gene polymorphisms.

Statistical analysis

The Mann-Whitney test was used to compare the data with *H. pylori* infection levels. Multiple comparisons were performed using the Kruskal-Wallis test, followed by the Mann-Whitney *U*-test with Bonferroni correction. The relation between histological findings and the production of IL-1β was assessed by Spearman's rank correlation coefficient.

Results

The genotype frequencies are listed in Table 1. The frequencies of *IL-1B*-511C/C, C/T, and TT were 24/99, 53/99, and 22/99, respectively. The frequencies of *IL-1B*-31C/C, C/T and T/T were 22/99, 53/99, and 24/99, respectively. *IL-1B*-31C/C was inversely associated with *IL-1B*-511T/T. The mean age was not different among the *IL-1B* gene polymorphism groups.

Gastric mucosal levels of IL-1β

H. pylori was detected in 64 patients. The gastric mucosal IL-1β levels in the corpus, but not those in the antrum were significantly higher in *H. pylori*-positive

Table 1. Genotype frequency according to disease presentation

		IL-1B-511		
Disease	Number	T/T *n*	C/T *n*	C/C *n*
Population studied	99	22 (22.2%)	53 (53.5%)	24 (24.2%)
Chronic gastritis	39	9	19	11
Duodenal ulcer	12	3	7	2
Gastric ulcer	17	2	9	6
Gastric cancer	6	2	2	2
Gastric polyp	3	0	3	0
Gastric adenoma	3	0	3	0
Nonulcer dyspepsia	19	6	10	3

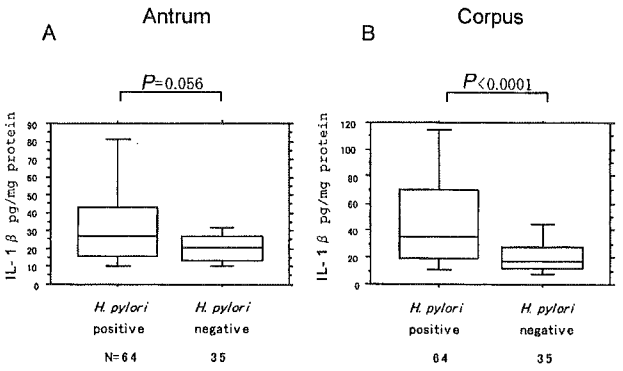

Fig. 1A,B. Gastric mucosal interleukin-1β (*IL-1β*) levels in patients with *Helicobacter pylori* infection. **A** IL-1β levels in the antrum. **B** IL-1β levels in the corpus

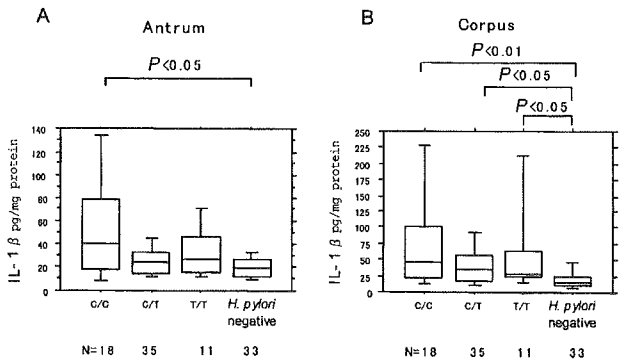

Fig. 2A,B. Gastric mucosal IL-1β levels in the antrum **A** and corpus **B** in relation to the genotypes at *IL-1B*-511

patients than in *H. pylori*-negative patients (Fig.1). No difference in IL-1β levels was observed between *H. pylori*-negative patients and those who had undergone successful eradication of *H. pylori*. In addition, no significant difference was observed between IL-1β levels in *H. pylori*-negative patients, regardless of *IL-1B* polymorphism. The gastric mucosal IL-1β levels in the antrum were significantly higher in genotype *IL-1B*-511C/C than in *H. pylori*-negative patients ($P < 0.05$; Fig. 2). In contrast, the IL-1β levels in the corpus were higher in *H. pylori*-positive patients than in *H. pylori*-negative patients, regardless of the *IL-1B* polymorphism.

Effects of IL-1B-*511 polymorphism on gastritis and atrophy scores*

Histopathological grading of antral and corpus biopsies was available for 87 of the 99 subjects with *H. pylori* infection. The *IL-1B* polymorphism had no influence on the degree of gastric neutrophil and mononuclear cell infiltration (Fig. 3). Furthermore, the polymorphism had no effect on the density of *H. pylori* or gastric atrophy.

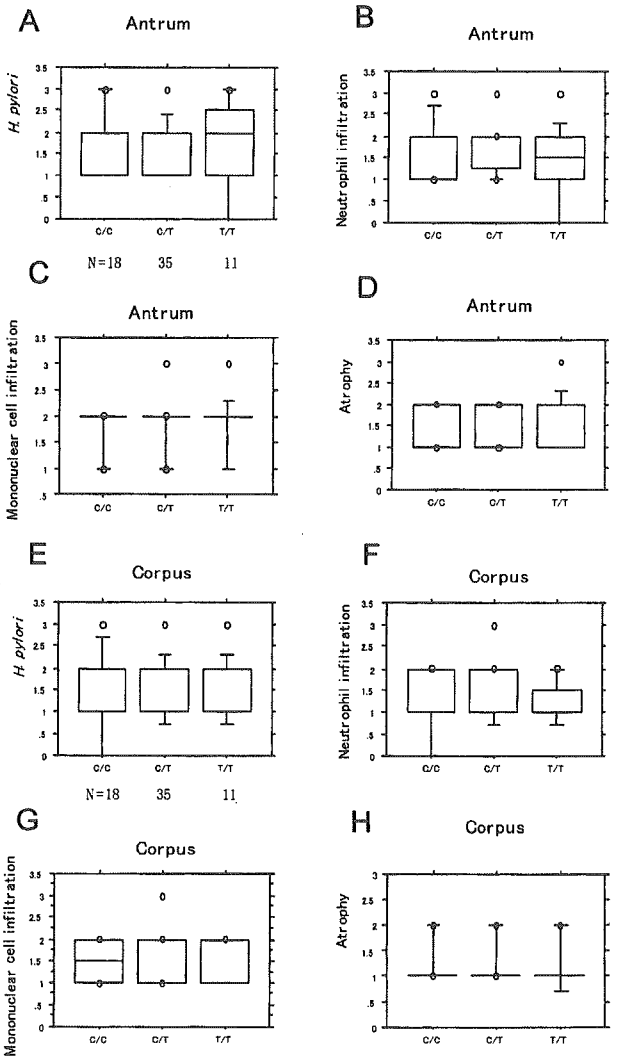

Fig. 3A–H. Median scores for histological gastritis in relation to *IL-1B* genotypes. **A** *H. pylori* density, **B** neutrophil infiltration, **C** mononuclear cell infiltration, and **D** atrophy in the antrum. **E** *H. pylori density*, **F** neutrophil infiltration, **G** mononuclear cell infiltration, and **H** atrophy in the corpus

IL-1β production and histological findings (Figs. 4 and 5).

The mucosal levels of IL-1β in both the antrum and corpus correlated with the density of *H. pylori*. The mucosal levels of IL-1β in both the antrum and corpus were higher in patients with severe neutrophil infiltration than in those with no infiltration. The mucosal levels of IL-1β in both the antrum and corpus were higher in patients with severe mononuclear cell infiltration than in patients with mild infiltration. The mucosal levels of IL-1β in the corpus, but not those in the antrum, were correlated with atrophy.

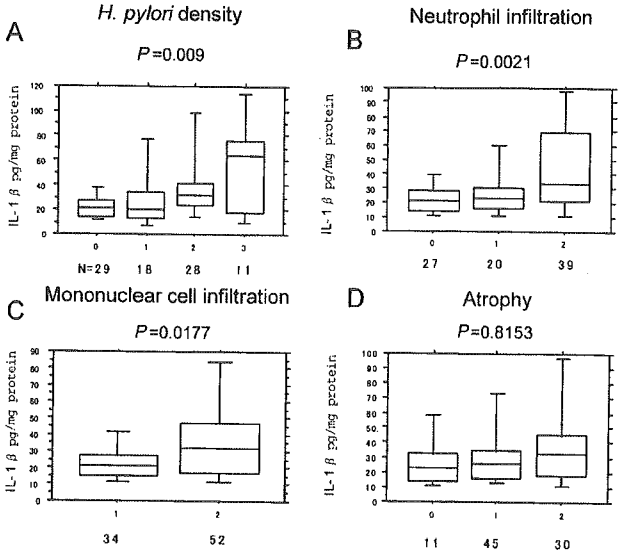

Fig. 4A–D. Relationship between IL-1β and histological gastritis in the antrum. **A** *H. pylori* density, **B** neutrophil in filtration, **C** mononuclear cell infiltration, and **D** atrophy in relation to IL-1β level

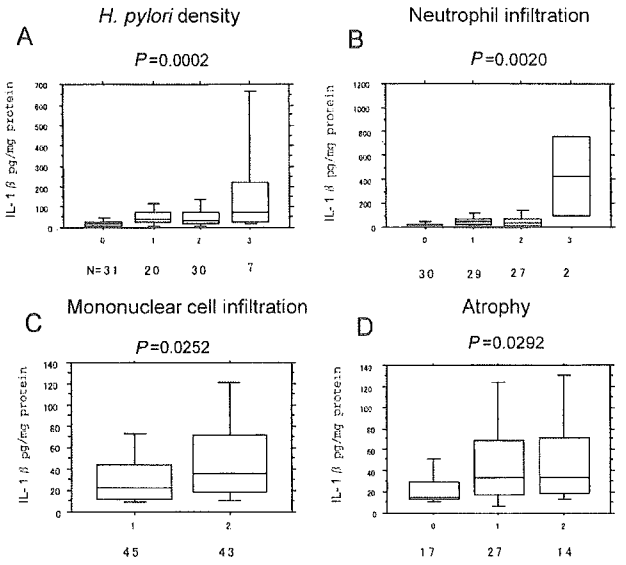

Fig. 5A–D. Relationship between IL-1β and histological gastritis in the corpus. **A** *H. pylori* density, **B** neutrophil in filtration, **C** mononuclear cell infiltration, and **D** atrophy in relation to IL-1β level

Discussion

On a global scale, gastric cancer remains the world's second most common malignancy. The discovery of *H. pylori* in the early 1980s has proved to be a turning point in the understanding of this malignancy. It is widely accepted that chronic *H. pylori* infection induces hypochlorhydria and gastric atrophy, both of which are precursors of gastric cancer.[14] IL-1β is a potent proinflammatory cytokine and is upregulated in the presence of *H. pylori*.[5,6] We confirmed, in a review of previous reports, that gastric mucosal IL-1β levels were significantly higher in *H. pylori*-positive patients than in-negative patients.[5,6,15] In the present study, the mucosal levels of IL-1β protein correlated significantly with the density of *H. pylori* in both the antrum and corpus.

Recently, El-Omar et al.[12] reported that *IL-1B* gene polymorphisms (*IL-1B*-511TT) and *IL-1RN**2/2* were associated with an increased risk of hypochlorhydria and gastric cancer. Machado et al.[16] also reported that carriers of *IL-1B*-511T and *IL-1RN**2/2* had an increased risk for developing intestinal-type gastric cancer, with odds ratios of 2.7 and 3.1, respectively.

In the present study, we confirmed previous data showing that the *IL-1B*-31C-to-T base transition was inverted in association with the –511T-to-C base transition.[17] The gastric mucosal IL-1β level in the antrum, but not that in the corpus, was significantly higher in the *IL-1B*-511C/C genotype than in *H. pylori*-negative patients ($P < 0.05$). The *IL-1B* polymorphism had no effect on the degree of gastric neutrophil or mononuclear cell infiltration. Furthermore, the polymorphism did not influence the density of *H. pylori* and gastric atrophy. Our finding that gastric mucosal IL-1β levels were higher in the *IL-1B*-511C/C genotype are not accord with the finding of Hwang et al.[18] that carriers of the T/T genotype had significantly higher IL-1β levels than carriers of the C/T genotype and those of the C/C genotype. The reason for this difference is unclear, although one possible explanation is a difference in the composition of the two study populations. Unlike the present study, their study cohort included 19 patients with gastric carcinoma and 34 patients with duodenal ulcer. Because patients with duodenal ulcer may have high acid secretion, the grade of corpus gastritis and cytokine production may be mild in patients with duodenal ulcers. The possibility of a type-II error in our study cannot be excluded, because of the small sample number; however, recently, Rad et al.[19] reported that gastric mucosal IL-1β levels, measured by quantitative reverse transcription (RT)-PCR were not different between *IL-1B-511*C/C and T groups.

Furthermore, conflicting data have been reported regarding the association between *IL-1B* polymorphisms and gastric cancer risk in the Japanese population. Severe corpus inflammation and atrophy are associated with a reduction in the pepsinogen (PG) I level and in the PG I/II ratio. In the present study, *IL-1B* polymorphisms had no effect on either histological gastritis or gastric atrophy. Furuta et al.[20] reported that the median serum PGI/II ratio was significantly lower in *H. pylori*-

infected subjects with the *IL-1B* T/T genotype compared with those with the C/T or C/C genotypes. They also showed that histologically severe inflammation in both the corpus and antrum was more common with the T/T or C/T genotypes than with the C/C genotype. In contrast, Matsukura et al.[21] analyzed the polymorphisms in Japanese, Chinese, Thai, and Vietnamese patients, and found that the *IL-1B* polymorphisms did not differ among the four populations, but that in patients with severe mucosal atrophy, the C/C polymorphism was dominant in the Japanese population, whereas the T/T + T/C polymorphisms were dominant in the Chinese population. The compositions of the patient populations may explain this difference. Further analysis of chronic gastritis, separate from that of duodenal ulcers is required.

Furthermore, Kato et al.[22] reported no association between *IL-1B* specific genotypes and gastric cancer in a Japanese population. This difference from the results reported by El-Omar may be explained by the difference in the haplotype frequencies of *IL-1B* and *IL-1RN* gene polymorphisms between the Japanese population and Western populations. Santtila et al.[23] found that the *IL-1RN**2 allele was associated with increased in vitro production of IL-1β. Machado et al.[16] reported that the frequencies of the *IL-1RN**2 allele in their controls and gastric cancer subjects were 20/220 and 24/152, respectively. In contrast, Hamajima et al.[17] reported that the frequency of *IL-1RN*2*2* was 1/241 in Japanese subjects. Thus, *IL-1RN* may play a more important role in the development of gastric cancer in Western countries than in Japan.

A recent prospective study by Uemura et al.[24] showed that gastric cancer developed in 36 of 1246 *H. pylori*-infected patients but in none of the 280 uninfected patients. They also reported that, among patients with *H. pylori* infection, those with severe gastric atrophy, corpus-predominant gastritis, and intestinal metaplasia were at a higher risk for gastric cancer. Suppression of acid secretion leads to *H. pylori* redistribution to the corpus, and, hence, gastric atrophy.[25] Because IL-1β is a potent inhibitor of gastric acid secretion, IL-1β production may be enhanced through the redistribution of *H. pylori*. In the present study, we showed that gastric IL-1β levels correlated with gastric atrophy in the corpus, regardless of *IL-1B* polymorphisms. These data suggested that other factors might regulate the production of IL-1β in the corpus of the stomach, independent of *IL-1B* polymorphisms. These findings provide further evidence of the lack of association between an *IL-1B* specific genotype and gastric cancer in the Japanese population.

In conclusion, *IL-1B* polymorphisms enhanced IL-1β production in the antrum; however, IL-1β production in the corpus was independent of *IL-1B* polymorphisms. These data suggested that other factors might regulate the production of IL-1β in the corpus of the stomach, regardless of *IL-1B* polymorphisms and high IL-1β production may be associated with the grade of gastric atrophy in the corpus mucosa in people with *H.pylori* infection.

References

1. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984;I: 1311–5.
2. Blaser MJ. Hypothesis on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology 1992;102:720–7.
3. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med 1991;325:1127–31.
4. Crabtree JE. The role of cytokines in *Helicobacter pylori* infection. Mucosal Immunol. Update 1994;2:5–7.
5. Noach LA, Bosma NB, Jansen J, Hoek FJ, van Deventer SJ, Tytgat GN. Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with *Helicobacter pylori* infection. Scand J Gastroenterol 1994;29:425–9.
6. Peek RM, Miller GG, Tham KT, Perez-Perez GI, Zhao X, Atherton JC, et al. Heightened inflammatory response and cytokine expression in vivo to cagA+ *Helicobacter pylori* strains. Lab Invest 1995;71:760–70.
7. Beales IL, Calam J. Interleukin 1 beta and tumour necrosis factor alpha inhibit acid secretion in cultured rabbit parietal cells by multiple pathways. Gut 1998;42:227–34.
8. Clark BD, Collins KC, Gandy MS, Webb AC, Auron PE. Genomic sequence for human prointerleukin 1 beta: possible evolution from a reverse transcribed prointerleukin 1 alpha gene. Nucleic Acids Res 1986;14:7897–914.
9. Arend WP. Interleukin 1 receptor antagonist. J Clin Invest 1991;88:1445–51.
10. Pociot F, Molvig J, Wogensen L. A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro. Eur J Clin Invest 1992;22:396–402.
11. Mansfield JC, Holden H, Tarlow JK, Di Giovine FS, McDowell TL, Wilson AG, et al. Novel genetic association between ulcerative colitis and the anti-inflammatory cytokine interleukin-1 receptor antagonist. Gastroenterology 1994;106:637–42.
12. El-Omar EM, Carrington M, Chow W-H, McColl KEL, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398–402.
13. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The Updated Sydney System. Am J Surg Pathol 1996;20:1161–81.
14. Sipponen P. Gastric cancer—a long-term consequence of *Helicobacter pylori* infection? Scand J Gastroenterol 1994;201: (Suppl)24–7.
15. Yamaoka Y, Kodama T, Kita M, Imanishi M, Kashima K, Graham DY. Relationship between clinical presentation, *Helicobacter pylori*, interleukin 1β and 8 production, and cagA status. Gut 1999;45:804–11.
16. Machado JC, Pharoah P, Sousa S, Carvalho R, Oliveira C, Figueiredo C, et al. Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. Gastroenterology 2001;121:823–9.
17. Hamajima N, Matsuo K, Saito T, Tajima K, Okuma K, Yamao K, et al. Interleukin 1 polymorphisms, lifestyle factors, and *Helicobacter pylori* infection. Jpn J Cancer Res 2001;92:383–9.
18. Hwang IL-Ran, Kodama T, Kikuchi S, Sakai K, Peterson LE, Graham DY, et al. Effect of interleukin 1 polymorphisms on

gastric mucosal interleukin 1B production in *Helicobacter pylori* infection. Gastroenterology 2002;123:1793–803.
19. Rad R, Dossumbekova Neu B, Lang B, Bauer S, Saur D, et al. Cytokine gene polymorphisms influence mucosal cytokine expression, gastric inflammation, and host specific colonization during *Helicobacter pylori* infection. Gut 2004;53:1082–9.
20. Furuta T, El-Omar EM, Xiao F, Shirai N, Takashima M, Sugimura H. Interleukin 1-B polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan. Gastroenterology 2002;123:92–105.
21. Matsukura N, Yamada S, Kato S, Tomtitchong P, Tajiri T, Miki M, et al. Genetic differences in *interleukin-1B* polymorphisms among four Asian populations: an analysis of the Asian paradox between *H. pylori* infection and gastric cancer incidence. J Exp Cancer Res 2003;22:47–55.
22. Kato S, Onda M, Yamada S, Matsuda N, Tokunaga A, Matsukura N. Association of *interleukin-1B* genetic polymorphism and gastric cancer risk in Japanese. J Gastroenterol 2001;36:696–9.
23. Santtila S, Savinainen K, Hurme M. Presence of IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1β production in vitro. Scand J Immunol 1998;47:195–8.
24. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
25. Kuipers ER, Lundell L, Klinkenberg-Knol EC, Havu N, Festen HPM, Liedman B, et al. Atrophic gastritis and *Helicobacter pylori* infection in patients with reflux esophagitis treated with omeprazole or fundoplication. N Engl J Med 1996;334:1018–22.

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Movement and Fixation of Intestinal Microbiota after Administration of Human Feces to Germfree Mice

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Human flora-associated (HFA) mice have been considered a tool for studying the ecology and metabolism of intestinal bacteria in humans, although they have some limitations as a model. Shifts in dominant species of microbiota in HFA mice after the administration of human intestinal microbiota was revealed by 16S rRNA gene sequence and terminal restriction fragment length polymorphism (T-RFLP) analyses. Characteristic terminal restriction fragments (T-RFs) were quantified as the proportion of total peak area of all T-RFs. Only the proportion of the T-RF peak at bp 366, identified as the *Gammmaproteobacteria* group and the family *Coriobacteriaceae*, was reduced in this study. Increased T-RFs over time at bp 56, 184, and 196 were affiliated with the *Clostridium* group. However, most of the isolated bacteria with unique population shifts were phylotypes. The vertical transmission of the intestinal microbiota of the mouse offspring was also investigated by dendrogram analysis derived from the similarity of T-RFLP patterns among samples. As a result, the intestinal microbiota of HFA mice and their offspring reflected the composition of individual human intestinal bacteria with some modifications. Moreover, we revealed that human-derived lactobacilli (HDL), which have been considered difficult to colonize in the HFA mouse intestine in previous studies based on culture methods, could be detected in the HFA mouse intestine by using a lactic acid bacterium-specific primer and HDL-specific primers. Our results indicate that the intestinal microbiota of HFA mice represents a limited sample of bacteria from the human source and are selected by unknown interactions between the host and bacteria.

Intestinal microbiota is composed of many kinds of bacteria. These indigenous intestinal bacteria play an important role in the health and disease of hosts, including nutrition, host immunity, and carcinogenesis. Experimental animal studies are indispensable when formulating basic concepts of the microbial ecology in the intestinal tracts of humans. However, the intestinal microbiota in experimental animals is quite different in composition from that in humans (23, 31). A recent study revealed the existence of many unidentified microorganisms, including some bacteria specific to the mouse intestine, by molecular techniques (2, 17, 35). Therefore, data obtained in animal experiments cannot be directly applied to humans.

Human flora-associated (HFA) mice have been considered a tool for studying the ecology and metabolism of human intestinal bacteria (6, 7, 30), although they have some limitations as a model (12). These limitations include differences in enzyme activity, concentrations of putrefactive products, and immunological activation by the composition of fecal bacteria (13, 15). Previous studies reported that 60% to 80% of the observable bacteria in human intestines could not be cultivated (10, 37). These limitations may therefore be caused by composition differences in microbiota of intestinal contents between humans and HFA mice. However, there are few reports about the use of molecular techniques with HFA mice or rats to determine the composition of microbiota including unidentified bacteria (5, 7, 15).

The genera *Lactobacillus* and *Bifidobacterium* are well known as beneficial bacteria for probiotics. These bacteria derived from humans seem to hardly colonize in the mouse intestine (12, 28, 41). In particular, it has been reported that lactobacilli have strong host specificity (24, 19, 39). The possibility has been considered that this phenomenon is caused by a difference in physiological condition and balance of microbes between humans and mice, but this has not been clarified.

In this study, 16S rRNA gene sequence and terminal restriction fragment length polymorphism (T-RFLP) analyses were used to reveal the shifts in the dominant bacteria of the intestinal microbiota in HFA mice after the administration of human fecal specimens. T-RFLP analysis is a useful molecular approach for the rapid assessment and comparison of diverse complex bacterial communities, such as those in soil, feces, and oral microbiota (20, 32, 33, 34). The horizontal transmission of intestinal microbiota in their offspring was also investigated. Moreover, lactobacilli were characterized, and the human-derived lactobacilli (HDL) were screened and detected in the intestines of HFA mice by use of genus-specific and the HDL-specific primers. This report provides new information regarding the intestinal microbiota of HFA mice.

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MATERIALS AND METHODS

Animals. Germfree BALB/c mice, from the Department of Infectious Diseases, Tokai University School of Medicine, Kanagawa, Japan, were used in all