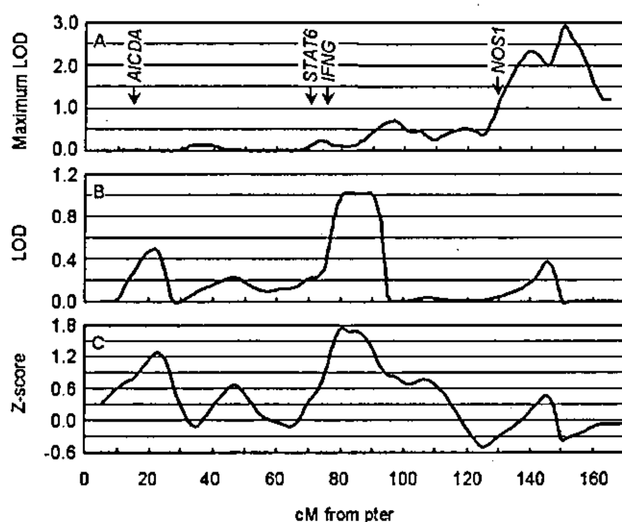

Fig. 1A–C Linkage results for asthma and total IgE level. All calculations were performed by GENEHUNTER 2. **A** Multipoint maximum likelihood (ML) estimate of identity by decent sharing for asthma affection status. **B** ML quantitative locus (QTL) variance estimation for $\log_{10}$ (total IgE level). ML QTL variance estimation (Kruglyak and Lander 1995) is performed instead of traditional Haseman-Elston (HE) QTL analysis because the values calculated with HE QTL analysis were affected by selection of sib pairs from the sib trio. **C** Nonparametric QTL analysis (Kruglyak and Lander 1995) for $\log_{10}$ (total IgE level)

for Dp-specific IgE. Thirty also showed atopic dermatitis. Results of the linkage analysis of the 18 microsatellite markers of chromosome 12 are shown in Fig. 1. A broad region from 135 cM to 160 cM exceeded 1.5 in MLS (Fig. 1A). Two peaks at 140 and 150 cM were observed, and the latter peak showed a MLS of 2.92, greater than the suggestive linkage level (Lander and Kruglyak 1995). Serum total IgE levels were $\log_{10}$ transformed and subjected to sib-pair quantitative locus (QTL) analysis. The result of maximum likelihood (ML) QTL variance estimation is shown in Fig. 1B. ML QTL variance estimation (Kruglyak and Lander 1995) was performed instead of the traditional Haseman-Elston (HE) QTL analysis, because the values with HE QTL analysis were affected greatly by the selection method of pairs from the sib trio. The highest peak of logarithm of odds (LOD) score (max = 1.02) was observed at 83 cM. No significant peaks were observed where linkage to asthma was observed. Nonparametric QTL analysis (Kruglyak and Lander 1995) showed the highest peak to be at 80 cM with a Z-score of 1.74 (P = 0.082) (Fig. 1C). The results of QTL analyses did not suggest evidence of QTL for $\log_{10}$ (total IgE level) on chromosome 12.

Association studies of genes on chromosome 12

Results of the case-control studies of chromosome 12 candidate genes are shown in Table 2. There were four types of alleles for the GT repeat of exon 1 of *STAT6* with the repeat number varying from 13 to 16 in our entire series. Comparison of allele frequencies between patients and controls showed a significant difference in the whole-allele distribution (P = 0.0054). This difference was primarily derived from the difference in the frequency of allele 3 (15 repeats). The frequency of this allele was significantly lower in the patients (Pc = 0.0044, odds ratio (OR) = 0.71, 95% confidence interval (95%CI) = 0.58–0.87). Other alleles did not show difference in frequency between controls and patients. Seven genotypes of this GT repeat were observed. The genotypic distribution differed between patients and controls (P = 0.0054). Homozygotes for allele 3 had a lower risk of asthma (Pc = 0.0035, OR = 0.718, 95%CI = 0.60–0.86).

We identified six alleles in the GT repeat polymorphism of intron 2 of *NOS1* (Table 2). The whole-allele distribution of this polymorphism of patients was significantly different from that of controls (P = 0.0082). This difference was primarily due to the difference in frequency of allele 3 (16 repeats) (Pc = 0.049, OR = 0.75, 95%CI = 0.60–0.93). Eleven genotypes of this GT repeat were observed. The whole genotypic distribution differed between patients and controls (P = 0.0019). Homozygotes for allele 3 had a lower risk for asthma than the other genotypes (Pc = 0.030, OR = 0.53, 95%CI = 0.33–0.84). There was a significant difference in the frequency of heterozygote for allele 4/5 between controls and patients (Pc = 0.028). The numbers of this genotype is small (zero in controls and six in patients), and its biological meaning is difficult to interpret.

Association of asthma was also tested for the CA repeat of the interferon γ gene (*IFNG*). We identified seven allele types and 16 genotypes of this polymorphism in the Japanese population (Table 2). There was no evidence of association between this polymorphism and asthma in our series.

We also investigated the SNP 465C/T of *AICDA*. Frequencies of the major allele were almost the same between controls and patients. Genotype distribution of controls was in Hardy-Weinberg equilibrium, whereas that of patients was not (χ^2 = 7.17, P = 0.027). We re-genotyped the patients and found no evidence of typing errors. Patients showed lower CT and higher TT genotype frequencies when compared to those of controls. However, these changes did not reach statistical significance in the association study (Table 2).

Discussion

We have found evidence of a suggestive linkage between 12q24.32–33 markers (144–155 cM) and childhood asthma in the Japanese population. The region identified was very close to the region reported by Wilkinson et al. (1998), who investigated linkage to their unique asthma score in the English population. In the genome-wide analysis of Japanese mite-sensitive childhood asthma, the highest MLS (1.92) was observed between D12S78 and D12S86 (110–132 cM) (Yokouchi et al. 2000). This

Table 2 Association study of the genes on chromosome 12

	Controls (frequency %)	Asthma (frequency %)	Significance level*
STAT6 exon 1 GT repeat			
Allelic distribution			
Allele 1 (13 repeat)	77 (22.4)	72 (31.6)	$Pc=0.061$
Allele 2 (14 repeat)	1 (0.3)	4 (1.4)	$Pc=0.34$
Allele 3 (15 repeat)	233 (67.7)	123 (53.8)	$Pc=0.0044$
Allele 4 (16 repeat)	33 (9.6)	29 (12.7)	$Pc>1.0$
Overall	344 (100.0)	228 (100.0)	$P=0.0032$
Genotypic distribution			
Allele 1/allele 1	14 (8.1)	12 (10.5)	$Pc>1.0$
Allele 1/allele 3	46 (26.7)	41 (36.5)	$Pc=0.81$
Allele 1/allele 4	3 (1.7)	7 (6.1)	$Pc=0.67$
Allele 2/allele 3	1 (0.6)	4 (3.5)	$Pc=0.59$
Allele 3/allele 3	81 (47.1)	30 (26.3)	$Pc=0.0035$
Allele 3/allele 4	24 (14.0)	18 (15.8)	$Pc>1.0$
Allele 4/allele 4	3 (1.7)	2 (1.8)	$Pc>1.0$
Overall	172 (100.0)	114 (100.0)	$P=0.0054$
NOS1 intron 2 GT repeat			
Allelic distribution			
Allele 1 (14 repeat)	2 (0.5)	1 (0.5)	$Pc>1.0$
Allele 2 (15 repeat)	11 (3.0)	2 (0.9)	$Pc=0.88$
Allele 3 (16 repeat)	189 (51.4)	88 (40.0)	$Pc=0.049$
Allele 4 (17 repeat)	3 (0.8)	7 (3.2)	$Pc=0.27$
Allele 5 (18 repeat)	126 (34.2)	97 (44.1)	$Pc=0.11$
Allele 6 (19 repeat)	37 (10.1)	25 (11.4)	$Pc>1.0$
Overall	368 (100.0)	220 (100.0)	$P=0.0082$
Genotypic distribution			
Allele 1/allele 3	2 (1.1)	1 (0.9)	$Pc>1.0$
Allele 2/allele 3	10 (5.4)	2 (1.8)	$Pc>1.0$
Allele 2/allele 5	1 (0.5)	0 (0.0)	$Pc>1.0$
Allele 3/allele 3	53 (28.8)	15 (13.6)	$Pc=0.030$
Allele 3/allele 4	3 (1.6)	1 (0.9)	$Pc>1.0$
Allele 3/allele 5	50 (27.2)	43 (39.1)	$Pc=0.42$
Allele 3/allele 6	18 (9.8)	11 (10.0)	$Pc>1.0$
Allele 4/allele 5	0 (0.0)	6 (5.5)	$Pc=0.028$
Allele 5/allele 5	28 (15.2)	18 (16.4)	$Pc>1.0$
Allele 5/allele 6	19 (10.3)	12 (10.9)	$Pc>1.0$
Allele 6/allele 6	0 (0.0)	1 (0.9)	$Pc>1.0$
Overall	184 (100.0)	110 (100.0)	$P=0.0019$
IFNG intron 1 CA repeat			
Allelic distribution			
Allele 1 (12 repeat)	39 (11.0)	24 (10.6)	$Pc>1.0$
Allele 2 (13 repeat)	209 (58.7)	110 (48.7)	$P=0.12$
Allele 3 (14 repeat)	6 (1.7)	7 (3.1)	$Pc>1.0$
Allele 4 (15 repeat)	91 (25.6)	78 (34.5)	$P=0.17$
Allele 5 (16 repeat)	7 (2.0)	3 (1.3)	$Pc>1.0$
Allele 6 (17 repeat)	0 (0.0)	1 (0.4)	$Pc>1.0$
Allele 7 (18 repeat)	4 (1.1)	3 (1.3)	$Pc>1.0$
Overall	356 (100.0)	226 (100.0)	$P=0.12$
Genotypic distribution			
Allele 1/allele 1	6 (3.4)	3 (2.7)	$Pc>1.0$
Allele 1/allele 2	19 (10.7)	8 (7.1)	$Pc>1.0$
Allele 1/allele 3	0 (0.0)	1 (0.9)	$Pc>1.0$
Allele 1/allele 4	8 (4.5)	9 (8.0)	$Pc>1.0$
Allele 2/allele 2	62 (34.8)	32 (28.3)	$Pc>1.0$
Allele 2/allele 3	3 (1.7)	0 (0.0)	$Pc>1.0$
Allele 2/allele 4	54 (30.3)	35 (31.0)	$Pc>1.0$
Allele 2/allele 5	6 (3.4)	2 (1.8)	$Pc>1.0$
Allele 2/allele 7	3 (1.7)	1 (0.9)	$Pc>1.0$
Allele 3/allele 3	0 (0.0)	2 (1.8)	$Pc>1.0$
Allele 3/allele 4	2 (1.1)	1 (0.9)	$Pc>1.0$
Allele 3/allele 7	1 (0.6)	1 (0.9)	$Pc>1.0$
Allele 4/allele 4	13 (7.3)	15 (13.3)	$Pc>1.0$
Allele 4/allele 5	1 (0.6)	1 (0.9)	$Pc>1.0$
Allele 4/allele 6	0 (0.0)	1 (0.9)	$Pc>1.0$
Allele 4/allele 7	0 (0.0)	1 (0.9)	$Pc>1.0$
Overall	178 (100.0)	113 (100.0)	$P=0.289$
AICDA 465C/T			
Allelic distribution			
C	214 (58.8)	119 (57.8)	
T	150 (41.2)	87 (42.2)	
Overall	364 (100.0)	206 (100.0)	$P=0.860$
Genotypic distribution			
C/C	63 (34.6)	41 (39.8)	
C/T	88 (48.4)	37 (35.9)	
T/T	31 (17.0)	25 (24.3)	
Overall	182 (100.0)	103 (100.0)	$P=0.099$

Table 2 (Continued)

* All significance levels were calculated by Fisher's exact methods. In allele-by-allele or genotype-by-genotype comparison, the *P* value was corrected by multiplying the number of alleles or genotypes and expressed as *Pc*

region is a little different from Wilkinson's and our peak regions. However, if we compare the region with MLS exceeding 1.0, the three regions overlap each other—our data 130–160 cM, Yokouchi et al. 110–150 cM, and Wilkinson et al. 130–160 cM. In an analysis of the Danish population, linkage with an MLS of 1.58 to atopic asthma at D12S392 (146 cM) was reported (Haagerup et al. 2002). More recently, linkage to airway responsiveness was also suggested at 147 cM by the Childhood Asthma Management Program (CAMP) study (Raby et al. 2003). Thus, all these studies shared a region from 140 to 150 cM. Several studies have reported linkage between asthma and the different regions of chromosome 12. The Collaborative Study on the Genetics of Asthma reported evidence of linkage to asthma of the 12q22 region. This study analyzed Caucasians, African Americans, and Hispanics and found a linkage peak at D12S2070 (125 cM) in Hispanics (CSGA 1997; Xu et al. 2001a). When the condition of loci of chromosome 14 was considered, this linkage peak became broader and greater and the region with an LOD score greater than 1.0 extended from 120 cM to the telomere (Xu et al. 2001a). The region detected in Barbados families was located at 12q21.1 (90 cM), which is clearly different from those found in the Japanese and English (Barnes et al. 1999; Barnes et al. 1996). Linkage to asthma of this region has also been suggested in German and Swedish populations (Wjst et al. 1999). A suggestive linkage (MLS = 2.81) for asthma was reported at a region around D12S390 (65 cM) in the Italian population (Malerba et al. 2000). The region was close to the region suggested by a CAMP study (Raby et al. 2003). In a founder population, Hutterites, Ober et al. (1998) suggested linkage of D12S375 (80 cM) to asthma in an earlier study, a finding not replicated in a more recent study (Ober et al. 2000). Chromosome 12 showed no evidence of linkage to asthma in Finnish families (Laitinen et al. 2001). A genome-wide study of the French population failed to detect a linkage to asthma but detected linkage to eosinophilia around the 130 cM region (Dizier et al. 2000). There was no evidence of

linkage to asthma-related phenotypes of chromosome 12 markers in the Chinese population, which is ethnically close to the Japanese population (Xu et al. 2001b).

In combination, the results of our and previous studies suggest that several genes located in 12q24–q33 are likely to be responsible for susceptibility to asthma. Some susceptibility genes of this region may be shared by several populations, and others may not. The MLS at the position of *NOS1* (128 cM), an association with which was detected in this study, was less than 1.0. The *NOS1* locus was 20 cM apart from the peak of the MLS. Thus, the region we detected in the present study most likely contains other asthma-susceptibility gene(s). In the 12q24 region, more than a hundred genes are listed in the Human Genome Map. Narrowing down the linked region by dense mapping and an intensive survey of SNPs in this region will be required to identify new susceptibility genes.

We investigated the existence of QTL for total IgE on chromosome 12 using asthma sib pairs. No region on chromosome 12 showed significant linkage to total IgE. Although type-2 error (false negative) could not be excluded due to the relatively small sample size, we speculate that loci in 12q22–q23 are asthma-susceptibility loci rather than QTLs that affect the total IgE level in the Japanese population. Using HE sib-pair QTL approaches, Barnes et al. (1996) reported that markers from 107 to 135 cM showed evidence of linkage to log (total IgE) in Barbados and Amish families. Xu et al. (2000) suggested linkage (LOD score = 2.73) to log(total IgE) of the region from *PAH* (108 cM) and D12S2070 (125 cM) in the Danish population using variance-component linkage analysis. Analysis of total IgE as a dichotomous trait (high versus normal IgE) was also performed by Nickel et al. (1997). They performed TDT in German children and presented evidence of association between markers located from 75 to 108 cM and high total IgE. On the other hand, many studies have shown negative linkage for total IgE. These include studies of the Australian population by Daniels et al. (1996), the Germans by Heinzmann et al. (2000a), the Germans and Swedish by Wjst et al. (1999), the Hutterites by Ober et al. (2000), the Chinese by Xu et al. (2001b), the French by Dizier et al. (2000), the Finnish by Leitinen et al. (2001), the Italians by Malerba et al. (2000), and the Danish by Haagerup et al. (2002). This inconstancy suggests that the relative importance of chromosome 12 loci in controlling total IgE varies from population to population.

We detected association of *STAT6* exon 1 GT repeat with Japanese childhood asthma. Our results clearly show association of the 15-repeat allele (allele 3) and asthma by allelic and genotypic distribution analyses. In Tamura's study using fewer samples than the present study, the number of heterozygote of allele 1 and allele 3 was significantly higher in patients with "allergic diseases" and that of homozygotes of allele 3 was lower in the patients with marginal statistical significance (Tamura et al. 2001). This observation was further confirmed by our present results: a change in the frequency of allele 3 homozygotes is more essential than that in the frequency of allele 1/allele 3 heterozygotes. Allele 3 appears to be a protective allele against the development of asthma in Japanese childhood asthma.

We also investigated the 2964G/A polymorphism in the 3'-UTR that was reported to be associated with adult asthma by Gao et al. (2000b) but failed to detect association in our samples (data not shown). Similar results were reported by Deutsch et al. (2002). These observations suggest that the *STAT-6* exon 1 GT repeat, but not 2964 G/A, is an asthma-susceptibility polymorphism. Further study is necessary to establish whether the polymorphism is functionally relevant to disease development or only a marker for the true functional polymorphism(s).

Association of the *NOS1* intron 2 GT repeat with asthma was also demonstrated in this study. Another study also describing the association of this polymorphism with asthma was carried out in the British population (Gao et al. 2000a). The 16-repeat allele of *NOS1* is a protective allele against asthma development in both British and Japanese populations, suggesting that this allele represents an old protective haplotype that evoked before the division of races. It is therefore possible that this polymorphism is associated with the disease in many ethnic groups. Moreover, Grasemann et al. (2000) have shown that *NOS1* exon 29 CA repeat in the 3'-UTR was associated with asthma in the US population. This is additional evidence that *NOS1* is a susceptibility gene for asthma in diverse populations. Again, further study is required to conclude whether these known polymorphisms are functionally relevant to the disease development or only markers for the true functional polymorphism(s).

We were unable to detect any association between *IFNG* and asthma in our series. Nakao et al. (2001) reported that allele frequency distribution differed between their 218 controls and 158 patients with atopic asthma in the Japanese population. Deviation of the distribution was primarily derived from the difference in frequency of allele 5 ($P = 0.0069$, $Pc = 0.048$). Frequencies in controls and patients of this allele were 4.8% and 1.3% respectively. In our study, the corresponding frequencies were 2.0% and 1.3% respectively. If we combine Nakao's and our data, P values become 0.012, which is not statistically significant after correction for the number of alleles ($Pc = 0.083$). More subjects are required to evaluate the significance of the association. Even if the association was confirmed, allele 5 would affect less than 5% of the population, i.e., the attributable fraction of this polymorphism would be low.

Although association of *AICDA* 465C/T (His155His) and asthma was suggested by TDT (Noguchi et al. 2001), our case-control study failed to prove the association of this polymorphism with asthma in the same population. In allelic distribution, allele frequency was essentially the same between the patient and control groups. Genotype distribution of the patients was not in

Hardy-Weinberg equilibrium. This did not result in significant difference in the genotype frequency between cases and controls. The data suggest that case samples were not well representative of the homogeneous population. We genotyped another set of 94 cases with this SNP. Genotypes CC, CT, and TT were 25, 50, and 19 cases respectively. Genotype frequency of the new set of patients was in Hardy-Weinberg equilibrium and not significantly different from the control value. Although we cannot exclude *AICDA* completely as a candidate gene for asthma, the effect of the 465C/T polymorphism on susceptibility to asthma was not as strong as the repeat polymorphisms of *STAT6* or *NOS1*.

In conclusion, we demonstrated that *STAT6* and *NOS1* loci are associated with childhood asthma in the Japanese population and showed evidence of "suggestive" linkage between region 12q24.23–q24.33 and asthma. This chromosome region most likely contains as yet unidentified asthma susceptibility gene(s).

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Identification of granulocyte subtype–selective receptors and ion channels by using a high-density oligonucleotide probe array

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Background: During inflammation, neutrophils, basophils, and eosinophils release cell type–specific mediators and proteases through signaling molecules, such as G protein–coupled receptors and ion channels. As such, ion channels and receptors, including G protein–coupled receptors, are common drug targets.

Objective: We sought to identify, for the first time, ion channels and receptors preferentially expressed by each granulocyte subtype.

Methods: Using GeneChip, we compared approximately 20,000 transcripts present in 7 leukocyte types, platelets, mast cells, and fibroblasts to identify granulocyte subtype–selective transcripts for receptors and ion channels. Granulocyte subtype–selective transcripts were chosen on the basis of several conditions, such as the transcript having a 5-fold or greater expression level compared with the maximum level of other leukocytes.

Results: Fifty-one transcripts were chosen to be preferentially expressed by each granulocyte subtype. Seventeen of the 51 transcripts have not been previously reported as granulocyte subtype selective. Among the 17 receptors and ion channels, 6 were basophil selective, eosinophil selective, or both and were not highly expressed by other organs, indicating that they might be potential targets for antiallergy drugs.

Conclusion: Use of this database of potential cell type–selective drug targets should minimize the efforts required for pharmaceutical development. (J Allergy Clin Immunol 2004;113:528-35.)

Key words: Basophils, eosinophils, granulocytes, G protein–coupled receptors, ion channels

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Three types of human blood granulocytes, eosinophils, basophils, and neutrophils, play roles in protecting against microbial infection by releasing cell type–specific mediators and proteases. Specifically, eosinophils and basophils evoke allergic reactions and damage nematodes.[1,2] As well as killing bacteria, neutrophils sometimes induce systemic vasculitis or multiple organ damage under certain conditions.[3,4] Thus targeting granulocyte type–selective functions is considered an important strategy for drug discovery.

Activation of blood granulocytes and tissue mast cells is generally characterized by an influx of extracellular calcium (Ca^{2+}), which is essential for subsequent release of granule-derived mediators, newly generated lipid mediators, and cytokines.[5] The mechanism by which granulocyte mediator secretion is sustained is therefore likely to include modulation of various types of ion channels. Flow of ions, including K^+ and Cl^-, might play an important role during granulocyte responses because they regulate cell membrane potential and thus influence Ca^{2+} influx.[6] Treatment of mast cells and basophils with pertussis toxin inactivates the Gi type of G proteins and abolishes degranulation induced by nonimmunologic ligands, such as thrombin and N-formylpeptide; however, it fails to inhibit the influx of Ca^{2+}.[7] Thus Ca^{2+}-independent stimulation of Gi is also involved in granulocyte degranulation. The thrombin (protease)–activated receptors and formylpeptide receptors are classified as G protein–coupled receptors (GPRs), having an extracellular N-terminal segment, a 7-transmembrane region that forms the transmembrane core, 3 exoloops, 3 cytoloops, and a C-terminal segment.[8] Thus ion channels and GPRs both play essential roles in degranulation, as well as other cellular functions important for granulocytes. As a result, both ion channels and GPRs are targets of drug development.[9]

As the human genome project nears completion, the identification of potential drug targets by using gene expression profiles from specific cell types is becoming practical and important for drug discovery.[10,11] The

Abbreviations used

AD: Average difference (of mRNA expression level)
Ca^{2+}: Extracellular calcium
EMR: Epidermal growth factor–like module containing mucin-like receptor
GAPDH: Glyceraldehyde-phosphate dehydrogenase
GPR: G protein–coupled receptor
PAR: Proteinase-activated receptor
Siglec: Sialic acid–binding Ig-like lectin

sequencing of the human genome is offering an unprecedented opportunity for the pharmaceutical development of drugs. Receptor genes and ion channel genes are found only in 5% and 1.3% of all genes present in the human genome, respectively.[10] However, receptors and ion channels are found in 45% and 5% of the molecular targets of all known drugs, respectively.[9,12,13] Thus receptors, including GPRs, and ion channels are now considered the most important drug targets.

Until recently, it has been impractical to analyze genome-wide expression of leukocytes. A newly developed technology, the microarray or high-density oligonucleotide probe array (GeneChip), is one of the latest breakthroughs in experimental molecular biology, which allows approximately 39,000 transcripts derived from a cell's transcriptome to be simultaneously monitored. Using this technology, we previously reported the transcriptome profiling of various types of mast cells and eosinophils.[14-16] In this study we used GeneChip (version U133A, containing approximately 22,000 gene probes) to examine the cell type–selective transcriptome expression of 7 types of leukocytes (basophils, eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, $CD14^+$ cells, and $CD19^+$ cells), platelets, mast cells, and fibroblasts. Then we focused on the expression of granulocyte-selective genes for ion channels, GPRs, and other receptors. We identified 17 novel granulocyte subtype–selective transcripts that might prove useful for drug development.

METHODS

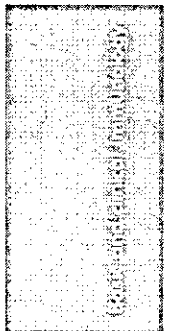
Purification of leukocytes

All human subjects in this study provided written informed consent, and the ethical review boards at the relevant hospitals (National Center for Child Health and Development and Jikei University School of Medicine) approved the study. The subjects used in this study were all healthy volunteers, specifically chosen for having no allergic diseases.

Granulocytes and mononuclear cells were separated from the venous blood of normal volunteers. Human basophils were semipurified by means of Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the cells were further purified by means of negative selection through use of a MACS Basophil Isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany), as described previously.[17] Eosinophils were isolated by using Percoll (1.090 g/mL) density centrifugation. The eosinophils were further purified by means of negative selection with anti-CD16–bound micromagnetic beads, as described previously.[18] Neutrophils were isolated by using Percoll (1.085 g/mL) density centrifugation and further purified by means of negative selection with anti-CD81 antibody and anti-mouse IgG-bound micromagnetic beads to eliminate contaminating eosinophils. These granulocytes purified from human peripheral blood were spun down onto glass slides by using the Cytospin II (Shandon Southern Instruments Inc, Sewickley, Pa). The purity of these cells was evaluated on the basis of 500 cells stained with May-Grünwald and Giemsa solutions.

For preparation of lymphocytes and monocytes, PBMCs were isolated by means of centrifugation on lymphocyte separation medium (Organon Teknika Corp, Durham, NC). Monocytes ($CD14^+$ cells) were prepared with magnetic bead–conjugated $CD14^+$ antibody (CD14 MicroBeads; Miltenyi Biotec) from PBMCs. $CD4^+$ and $CD8^+$ cells were also sorted by using magnetic bead–conjugated $CD4^+$ (CD4 MicroBeads; Miltenyi Biotec) and $CD8^+$ antibodies (CD8 MicroBeads; Miltenyi Biotec), respectively, from PBMCs after depletion of $CD14^+$ cells with MACS CD14 MicroBeads (Miltenyi Biotec). The purity of $CD4^+$, $CD8^+$, and $CD14^+$ cells was evaluated by staining the magnetic bead–conjugated cells compared with feasible control cell preparations, such as unpurified cells, with FITC-labeled goat anti-mouse Ig (BD Pharmingen, Tokyo, Japan). Peripheral B cells were purified by using a combination of negative (MicroBead-conjugated antibodies to CD3, CD7, CD14, CD42b, and CD56; Miltenyi Biotec) and positive (CD19 MicroBeads; Miltenyi Biotec) selection with MicroBeads (Miltenyi Biotec). Blood samples were mixed with 3.8% (wt/vol) sodium citrate solution (9:1) and centrifuged at 260*g* for 15 minutes at 20°C to obtain platelet-rich plasma. The plasma was centrifuged again at 260*g* for 15 minutes to remove any contaminating erythrocytes and leukocytes.

Human mast cells were derived from cord blood $CD34^+$ progenitor cells, as described previously.[11-14] Briefly, progenitor cells purified from peripheral blood by using $CD34^+$ isolation kits (Miltenyi Biotec) were cultured in Iscove modified Dulbecco medium supplemented with 1% insulin-transferrin-selenium supplements (Invitrogen, Tokyo, Japan), 50 μmol/L 2-mercaptoethanol, antibiotics, and 2% FCS in the presence of 100 ng/mL stem cell factor and 50 ng/mL IL-6. After 11 to 14 weeks of culture, tryptase-positive cells represented more than 99% of the cells.



Purification of human lung mast cells and nasal polyp–derived fibroblasts

Normal human lung tissue dissected during surgery was obtained macroscopically after obtaining informed consent. Human lung mast cells were dispersed from chopped lung specimens by means of an enzymatic procedure and were purified by using magnetic bead affinity selection with the mAb anti-kit YB5.B8 (BD PharMingen, San Diego, CA), as described previously.[19] The cells were further cultured in the presence of stem cell factor and IL-6 for several weeks. Human nasal polyp–derived fibroblasts were obtained as previously reported.[20]

GeneChip expression analysis

Human genome–wide gene expression was examined by using the Human Genome U133A probe array (GeneChip; Affymetrix, Santa Clara, Calif), which contains the oligonucleotide probe set for 22,000 full-length genes. Experiments were performed in accordance with the manufacturer's protocol (Expression Analysis Technical Manual) and previous reports.[11-14] Total RNA (1-5 μg) was extracted from 10^7 cells. Double-stranded cDNA was synthesized by using a SuperScript Choice system (Invitrogen) and a T7-(dT)24 primer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates by using a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The biotinylated cRNA was hybridized with a

probe array for 16 hours at 45°C. In some experiments, as indicated in the supplementary table, biotinylated cRNA was prepared by using 2 cycles of cDNA synthesis, and in vitro transcription for target amplification was performed according to the manufacturer's directions (Affymetrix, Inc). For the latter protocol, we used 100 ng of total RNA. After washing, the hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, Ore) and then scanned with an HP gene array scanner. The fluorescence intensity of each probe was quantified by using a computer program, GeneChip Analysis Suite 5.0 (Affymetrix). The expression level of single mRNA was determined as the average fluorescence intensity among the intensities obtained with 11 paired (perfect-matched and single nucleotide-mismatched) probes. If the intensities of mismatched probes were very high, gene expression was judged to be absent, even if a high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The level of gene expression was determined as the average difference (AD) by using the GeneChip software. Each AD level was then normalized by dividing it with the median value of 22,283 AD levels obtained in an experiment (ie, the normalized AD level).

Real-time RT-PCR

Total RNA was isolated with Isogen (Nippon gene, Tokyo, Japan), according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RNA was subsequently treated with DNase I (Invitrogen) reverse transcribed by using Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed with 10 ng of cDNA in 25 μL of final volume by using the primers and probes supplied by the Assays-on-Demand Gene Expression System (Applied Biosystem, Tokyo, Japan), according to the manufacturer's instructions. Measurement of gene expression was performed with the ABI PRISM 7700 Sequence Detector. The expression level of each gene was normalized to a reduced glyceraldehyde-phosphate dehydrogenase (GAPDH).

Staining of basophils with anti-HTm4

Basophils purified from human peripheral blood with the Basophil Isolation Kit (Miltenyi Biotec) were spun down onto glass slides by using the Cytospin II (Shandon). Cells were fixed with aceton for 1 minute and then blocked in 2% goat serum in 50 mmol/L Tris-Cl, pH 7.4, for 1 hour. Cells were further incubated for 2 hours with 2 μg/mL of the polyclonal antibody rabbit anti-hHTm4. Cells were then washed 3 times with PBS and incubated with a secondary antibody, highly cross-adsorbed Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L; Red; Molecular Probes), for 1 hour. After 3 PBS washes, air-dried cells were further mounted by using the Prolong Anti-Fade Kit (Molecular Probes). Slides were scanned with a Zeiss Laser Scanning Microscope 5 Pascal (Carl Zeiss Microimaging Inc, Thornwood, NY).

Purity and viability of the leukocytes and RNA quality

We used leukocyte samples in this study only if the purity of each cell type was at least 98%, but there were greater than 0.5% contaminated cells in any of the samples. We could not evaluate the purity of CD19$^+$ cells and platelets because of the lack of feasible controls or methods. However, specific transcript markers for non-granulocytes (eg, CD4, CD8, CD14, CD19, and IgG), as well as granulocyte subtype-specific transcripts, were reasonably expressed by each leukocyte type, as shown in Online Repository Table E1. Regarding the viability, we qualified the RNA before GeneChip assay by using Array Quality Metrics Comparisons Software (Affymetrix), as well as trypan blue staining (they were always >95% viable), because RNAse-rich granules derived from degenerating cells rapidly destroy RNA transcripts. Briefly, to evaluate the quality of RNA, the ratio of the 3′-probe set and 5′-probe set of housekeeping genes were compared as shown in Online Repository Table E1. According to the above software's guidance, the ratio of greater than 2:1 at a standard sample (5 μg total RNA) protocol and that of greater than 10:1 at a small sample (50 ng of total RNA) protocol were recommended. As shown in Online Repository Table E1, the cells used in the present study had the appropriate ratios of the 3′-probe set and 5′-probe set of housekeeping genes, suggesting that these cells were highly viable.

Statistical analysis

Because logarithmic normalized AD levels were normally distributed within each group, the unpaired, parametric, 2-tailed Student *t* test was used to analyze the data on a logarithmic scale.

RESULTS

In this study we have used a high-density oligonucleotide probe array (GeneChip) to measure the expression levels of approximately 20,000 different transcripts in highly purified cells. These cells were basophils, eosinophils, neutrophils, monocytes (CD14$^+$), T lymphocytes (CD4$^+$ and CD8$^+$ cells), B lymphocytes (CD19$^+$), lung-derived mast cells, cord blood–derived cultured mast cells, and nasal polyp–derived fibroblasts. The GeneChip assay allows the simultaneous measurement of large numbers of transcripts by using relatively small numbers of cells. Using this technology, we could even measure triplicate transcriptome levels of basophils, the most rare granulocytes in peripheral blood.

Cell type–selective transcripts were selected on the basis of the following criteria. First, the average normalized AD expression level of each gene in a certain cell type must be 3-fold or greater than the maximal level in other cell types, and second, it must be significantly ($P < .01$) greater than that in other cell types. Third, the AD expression level provided with "absence" or "marginal" call by GeneChip Suite Software should be observed only once or not at all in the 3 or 4 independent experiments (3 experiments for basophils and 4 experiments for eosinophils and neutrophils) by using different cell populations. Finally, for the transcripts preferentially expressed for the 2 different cell types, such as basophils and eosinophils, the average normalized AD expression levels in the 2 cell types should be within 3-fold of each other. By using these standards, we found 83 basophil-selective, 37 eosinophil-selective, 257 neutrophil-selective, 34 basophil/eosinophil-selective, 19 eosinophil/neutrophil-selective, and 17 basophil/neutrophil-selective transcripts. Because of the functional similarity with basophils, mast cell–selective transcripts were also examined, and 63 mast cell–selective and 11 mast cell/basophil–selective transcripts were also detected (Table I and Online Repository Table E1). Because mast cells, basophils, and eosinophils play similar roles in allergic inflammation, the transcripts preferentially expressed for the 3 granulocytes by comparing their average normalized AD levels with those of other leukocytes. Thirty-four transcripts were then selected; however, most of them were overlapped with the transcripts listed in

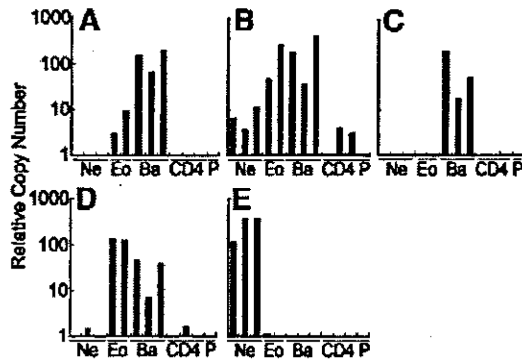


FIG 1. Real-time quantitative PCR analysis. The relative mRNA expression level by each cell type against PBMCs was shown after normalization of mRNA levels for HTm4 (0.42 per 100 GAPDH; **A**), Ca^{2+} receptor α 1D (0.003 per 100 GAPDH; **B**), prostaglandin E receptor 3a2 (0.12 per 100 GAPDH; **C**), EMR-1 (0.62 per 100 GAPDH; **D**), and aquaporin 9 (0.92 per 100 GAPDH; **E**), expressed by PBMCs. *Ne,* Neutrophils; *Eo,* eosinophils; *Ba,* basophils; *CD4,* $CD4^+$ cells; *P,* PBMCs.

Online Repository Table E1. Only 4 transcripts (*MYB, SAMSN1, BACE2,* and *CASP3*) were found not to be overlapped, and they were not receptors or ion channels.

Among the 491 granulocyte–selective transcripts listed in Online Repository Table E1, 4 ion channels, 19 GPRs, and 28 other receptors were further selected (Table II). When plural transcripts obtained by different probe sets had identical Genebank or Unigene accession numbers (http://www.ncbi.nlm.nih.gov/), the transcript showing the highest expression level was selected.

Ion channels and receptors preferentially expressed by granulocytes other than neutrophils

Eosinophils, basophils, and mast cells play an important role in the pathogenesis of allergic diseases but do not play an essential role in killing microbes, except for nematodes. On the other hand, neutrophils play a crucial role in killing microbes, such as bacteria. Caution should be taken in regulating neutrophil function, even in the case of neutrophil-induced inflammation. Therefore the molecules present only in granulocytes, except for neutrophils, would be important pharmaceutical targets for allergic disorders.[1,2]

Among the 51 granulocyte-selective transcripts for ion channels and receptors, we identified 17 granulocyte-selective transcripts that have not been reported for their selective expression (shown in bold letters in Table II). Of these 17 transcripts, 8 were preferentially expressed by granulocytes other than neutrophils. Among these 8 transcripts, the 2 transcripts for fibroblast growth factor receptor 2 and low-density lipoprotein receptor were found to be expressed by multiple tissue cell types (shown at http://www.lsbm.org/index_e.html, which displays genomic expression of 55 different human tissue cells, such as brain, heart, and lung cells, using the same experimental system [Affymetrix U133A] as ours). Thus they might not be suitable as a drug target because important organs that are unrelated to allergic inflammation (eg, the brain) express it. Among the 6 novel transcripts found to be preferentially expressed by granulocytes except for neutrophils, we focused on the following 4 transcripts expressed by granulocytes, including basophils. They were Ca^{2+} channel (*CACNA1D*), prostaglandin E receptor (*EP3A2*), epidermal growth factor–like module–containing mucin-like receptor (EMR) 1 (*EMR1*), and HTm4 (*MS4A3*).

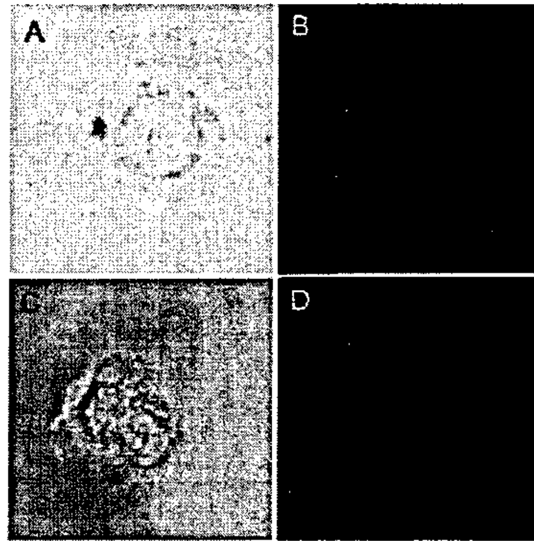


FIG 2. Demonstration of HTm4 protein on human basophils. Cells were incubated with 2 μg/mL polyclonal rabbit anti-hHTm4 antibody (**A** = phase contrast, **B**) or 2 μg/mL rabbit IgG (H+L) as a control (**C** = phase contrast, **D**), followed by incubation with a secondary antibody, highly cross-adsorbed Alexa Fluor 546–conjugated goat anti-rabbit IgG (H+L; Fig 2, **B** and **D**).

Basophils are the rarest granulocytes present in human peripheral blood, and as such, their complete transcriptional profiles remain unclear, and no basophil-selective transcripts have previously been reported.[21] Although eosinophils and mast cells have been considered important therapeutic targets for allergic diseases for a long time, recent studies suggest the importance of basophils in the pathogenesis of severe allergic diseases, such as fatal asthma.[2,22] Therefore we further examined the selective expression of these 4 basophil- or basophil/eosinophil-selective transcripts by using real-time RT-PCR. As shown in Fig 1, including a known molecule preferentially expressed by neutrophils, aquaporin 9,[23] the results obtained with the GeneChip assay were confirmed by using this highly accurate and reproducible method.[24]

Among these 4 molecules, we could obtain a suitable antibody against HTm4, which is a member of a family of 4-transmembrane proteins that includes CD20 and the FcεRI β-chain.[25] Genetics provided evidence for the existence of multiple loci relevant to atopic asthma on chromosome 11q13, including HTm4.[26] Most recently, we have published data identifying HTm4 as a hematopoietic cell-cycle regulator.[27] Using specific antibody against HTm4, we could detect the expression of HTm4 at the protein level in basophils (Fig 2). The

TABLE I. Representative cell type–selective transcripts in granulocytes

Accession no.*	Transcript	SI†	Normalized AD level
Basophil selective			
NM_000589.1	IL-4	73.3	13.3
L35848.1	HTm4	38.2	132.1
BC005912.1	FcεRIα	12.7	219.0
Eosinophil selective			
NM_001140.1	Arachidonate 15-lipoxygenase	74.1	18.3
NM_024703.1	FLJ22593	19.1	29.1
NM_014442.1	Siglec-8	9.8	16.9
Neutrophil selective			
NM_004633.1	IL-1R, type II	127.9	51.5
U73191.1	Inward rectifier K^+ channel Kir1.3	107.5	98
NM_001557.1	CXCR2 (IL-8Rβ)	39.3	105.2
Mast cell selective			
AF206667.1	Tryptase β	84.3	159.4
NM_001911.1	Cathepsin G	51.5	72.1
BC005929.1	Major basic protein	31.6	72.5
Basophil/eosinophil selective			
M75914.1	IL-5Rα	42.8	19.4 (B), 29.3 (E)
NM_004778.1	CRTH2	16.6	23.9 (B), 38.1 (E)
NM_001828.3	Charcot-Leyden crystal protein	15.2	229.2 (B), 198.6 (E)
Eosinophil/neutrophil selective			
NM_005306.1	GPR 43 (PAR1-like)	21.7	11.7 (E), 32.9 (N)
NM_004668.1	DHHC domain containing 18	6.6	16.2 (E), 44.5 (N)
Basophil/neutrophil selective			
NM_016006.1	CGI-58 protein	5.8	12.6 (B), 21.2 (N)
Basophil/mast cell selective			
NM_001870.1	Carboxypeptidase A3	59.2	111.7 (M), 137.3 (B)
NM_002529.2	TRK neurotrophin receptor	34.7	3.1 (M), 7 (B)
NM_000139.1	FcεRI β	21.2	22.2 (M), 43.8 (B)

*GenBank accession number (http://www.ncbi.nlm.nih.gov). †The selectivity index *(SI)* was calculated by comparing the normalized AD level in a cell type or the normalized AD level of 2 cell types with the maximal gene expression level of the other 8 or 9 cell types. The complete list of the genes having an SI of greater than 3 is shown in Online Repository Table E1.

amount of molecules expressed by various cell types should be quantified, and the effect of any identified antagonists should be tested on the cell types found to express these molecules to confirm whether these ion channels and receptors could be potential drug targets for diseases involving basophil activation.

As has been well documented and expected, IL-3 receptors, IL-5 receptors, chemokine receptor CCR3,[1,2] sialic acid–binding Ig-like lectin (Siglec)–8,[28] Siglec-6,[15] histamine H4 receptor,[29] and chemoattractant receptor–homologous molecule expressed on T_H2 cells (CRTH2)[30] were preferentially expressed by basophils, eosinophils, or both or mast cells. These consistent observations strengthened the reliability of the present methods and research strategy. Indeed, the antibody against Siglec-8 can induce selective apoptosis of eosinophils and is expected to be useful therapeutically.[28]

Ion channels and receptors preferentially expressed by granulocytes, including neutrophils

Of the 17 transcripts that have not been reported as granulocyte selective, 9 were preferentially expressed by granulocytes, including neutrophils. Pharmaceutical targets of selective granulocyte transcripts should treat inflammatory diseases without affecting the function of important organs that are unrelated to inflammation, as well as the function of lymphocytes. However, 4 of the 9 neutrophil-selective transcripts were expressed by multiple organ tissues. One of the 4 neutrophil-selective transcripts encoded proteinase-activated receptor (PAR)–2, a receptor for mast cell tryptase that is linked to the pathogenesis of allergic diseases, such as asthma.[31,32] PAR-2 transcripts are also abundantly expressed by tissue types, including skin and intestine, which are often the target organs for allergic diseases. But the development of PAR-2 antagonists for use as antiallergic drugs might be unsuitable because it might downregulate neutrophil function and thereby induce bacterial infection.

DISCUSSION

We identified 51 granulocyte-selective genes for ion channels and receptors by examining approximately 20,000 kinds of transcripts derived from 16,000 genes from 10 different types of cells with the U133A GeneChip, which covers approximately half of the genes present in the human genome. The majority of these transcripts encoded molecules known or expected to be granulocyte subtype–selective, such as the IL-3 receptor and Fcε receptors.

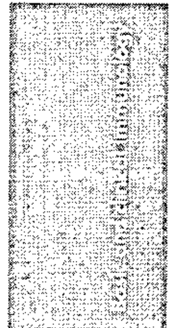
TABLE II. Granulocyte subtype–selective transcripts for ion channels and receptors

Transcript (GenBank accession no.)	Cell type*	MC	Ba	Eo	Ne	Pl	CD4	CD8	CD14	CD19	Fb	TC†
Ion channels												
Ca^{2+} channel type A1 D (BE550599)	Ba, Eo	0.1	1.7	1.5	0.4	0.0	0.3	0.1	0.0	0.3	0.0	*0*
Aquaporin 9 (NM_020980.2)	Ne	0.7	0.1	0.4	137.4	0.4	0.9	0.1	8.6	0.1	0.2	*7*
K^+ channel Kir1.3 (U73191.1)	Ne	0.9	0.2	0.4	99.5	0.7	0.2	0.0	0.8	0.0	0.0	*5*
K^+ channel Kir2.1 (AF153820.1)	Ne	0.7	3.8	5.3	40.7	0.4	0.2	0.5	1.5	0.7	1.1	*6*
GPR												
Histamine H_4 R (AF312230.1)	Ba	0.7	34.2	9.4	0.7	0.4	0.8	0.5	0.6	0.0	0.1	*0*
PGE R type 3a2 (X83858.1)	Ba	0.8	10.3	0.1	0.7	0.6	0.3	0.2	0.2	0.5	1.7	*0*
C3a R (U62027.1)	Ba, Eo	11.8	55.7	39.4	2.0	1.6	1.6	1.5	3.0	0.6	0.5	*3*
CCR3 (NM_001837.1)	Ba, Eo	0.6	117.4	90.9	24.9	0.2	0.5	0.4	0.2	0.2	0.4	*0*
CRTH2 (NM_004778.1)	Ba, Eo	1.1	26.0	38.2	2.0	0.8	1.4	1.0	1.2	0.9	0.5	*0*
EMR-1 (NM_001974.1)	Ba, Eo	0.8	33.5	90.9	4.2	3.4	1.7	0.8	7.1	1.6	0.5	*1*
Adenosine A_3 R (NM_000677.2)	Eo	2.6	2.9	15.4	2.3	1.2	1.6	0.5	2.0	0.5	0.5	*0*
P2Y2 purinergic R (NM_002564.1)	Eo	0.1	0.1	5.5	0.1	0.2	0.1	0.3	1.2	0.2	0.1	*0*
GPR105 purinergic R (NM_014879.1)	Eo	2.6	2.9	15.4	2.3	1.2	1.6	0.5	2.0	0.5	0.5	*2*
GPR, Edg-4 (AF011466.1)	Eo, Ne	1.3	2.8	15.9	24.2	0.1	3.6	5.0	3.8	1.9	0.9	*2*
PAR1-like GPR43 (NM_005306.1)	Eo, Ne	0.3	0.7	12.4	35.2	0.8	0.1	0.1	0.7	0.4	0.1	*0*
C5a R (NM_001736.1)	Ne	2.3	21.6	13.6	92.6	2.3	1.2	0.5	25.6	1.0	0.4	*5*
CXCR1 IL-8R (NM_000634.1)	Ne	0.2	4.3	0.3	83.4	0.4	0.3	0.2	0.2	0.1	0.1	*0*
CXCR2 IL-8R (NM_001557.1)	Ne	0.2	1.1	1.5	112.1	2.7	0.3	0.8	0.7	0.6	0.0	*1*
Formyl peptide R 1 (NM_002029.1)	Ne	2.8	23.5	8.8	282.9	3.6	1.3	0.7	62.6	1.1	0.5	*0*
Formyl peptide R 2 (U81501.1)	Ne	0.4	0.5	0.6	75.5	0.1	1.0	0.6	6.0	0.8	0.1	*5*
GPR77 (NM_018485.1)	Ne	0.1	0.9	0.8	3.4	0.0	0.3	0.2	1.0	0.1	0.3	*2*
GPR86 purinergic R (NM_023914.1)	Ne	0.3	0.2	17.9	88.2	1.9	0.1	0.2	12.3	0.6	0.0	*1*
PAR2 (BE965369)	Ne	0.1	0.3	2.2	36.2	0.1	0.7	0.1	1.6	0.4	1.2	*15*
Other receptors (R)												
FcεRIα (BC005912.1)	Ba	19.4	219.0	4.6	5.5	1.4	5.1	0.8	1.4	0.4	0.2	*0*
HTm4 (L35848.1)	Ba	0.6	133.1	6.9	3.5	0.2	0.5	0.4	0.1	0.2	0.0	*1*
IL-3 R (NM_002183.1)	Ba	0.6	52.6	2.0	0.7	0.2	0.3	0.2	0.7	0.1	0.1	*0*
CD244 NK cell R (NM_016382.1)	Ba, Eo	0.3	56.0	16.5	1.2	0.1	0.4	3.5	5.2	1.1	0.1	*0*
Fibroblast growth factor R 2 (NM_022969.1)	Ba, Eo	0.1	27.9	12.1	0.2	0.1	0.1	0.2	0.1	0.1	1.0	*17*
IL-5R α (M75914.1)	Ba, Eo	0.9	20.5	30.4	0.6	0.1	0.1	0.1	0.1	0.2	0.1	*0*
Siglec-8 (NM_014442.1)	Eo	1.8	0.3	17.4	0.4	0.3	0.2	0.1	0.2	0.5	0.2	*0*
CD117 c-KIT (NM_000222.1)	MC	89.0	7.2	4.2	1.2	0.8	0.2	0.5	0.1	0.1	0.5	*15*
Siglec-6 (D86358.1)	MC	5.6	0.2	0.0	0.2	0.5	0.0	0.0	0.0	0.4	0.0	*0*
FcεRIβ (NM_000139.1)	MC, Ba	22.3	44.3	0.4	0.6	1.6	0.5	0.2	0.0	0.3	0.1	*0*
Low-density lipoprotein R (NM_000527.2)	MC, Ba	20.7	20.4	1.3	1.8	2.3	2.1	3.4	3.5	0.8	6.3	*34*
Tropomyosin-related kinase neurotrophin R (NM_002529.2)	MC, Ba	4.6	7.3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	*0*
Butyrophilin-like R (AK025267.1)	Ne	1.1	1.7	1.5	6.9	1.5	1.7	1.6	0.9	1.5	1.3	*1*
CD120a, TNF-R-I (NM_001065.1)	Ne	1.7	1.2	7.8	74.7	1.3	2.2	5.9	17.4	0.6	16	*35*
CD95, Fas, APO-1 (AA164751)	Ne	1.4	10.9	7.3	50.4	1.3	5.6	4.8	3.6	2.6	9.7	*7*
Decoy R1, TRAILR3 (AF012536.1)	Ne	0.1	1.5	7.7	78.7	0.5	0.2	0.1	0.7	0.1	0.6	*0*
FcγRIIc2 (U90939.1)	Ne	1.5	2.4	7.8	59.9	0.3	0.2	0.1	7.2	2.3	0.2	*2*
FcγRIIc3 (U90940.1)	Ne	2.4	10.7	10.0	84.3	3.0	1.4	0.6	14.0	7.2	1.0	*5*
FcγRIII (J04162.1)	Ne	0.7	1.6	1.9	199.6	6.6	1.3	1.3	2.2	2.6	0.1	*4*
G-CSF R (NM_000760.1)	Ne	0.1	0.4	1.6	163.6	0.2	0.8	0.2	25.5	0.1	0.2	*3*
IL-13R (U81379.3)	Ne	0.3	0.2	2.0	14.1	0.4	0.4	0.3	2.9	1.3	1.3	*0*
IL-1R, type II (NM_004633.1)	Ne	0.1	0.1	0.1	53.5	0.2	0.4	0.1	0.2	0.0	0.0	*3*
Insulin-like growth factor R1 (NM_000875.2)	Ne	0.3	3.5	5.0	17.4	1.6	0.1	2.8	2.4	2.0	3.5	*3*
Insulin-like growth factor R2 (NM_000876.1)	Ne	4.6	0.9	5.4	85.3	1.7	2.9	8.7	8.4	4.8	15	*32*
Leukocyte Ig-like R A2 (NM_006866.1)	Ne	0.5	5.8	4.3	41.2	1.8	0.0	0.1	11.6	0.5	0.1	*2*
Toll-like R1 (AL050262.1)	Ne	0.6	0.3	1.2	31.5	1.6	0.8	0.7	3.0	1.5	0.3	*0*
Toll-like R2 (NM_003264.1)	Ne	0.9	6.0	1.3	83.8	1.6	1.3	0.1	26.3	0.9	0.4	*0*
Toll-like R6 (NM_006068.1)	Ne	0.5	1.0	0.9	8.8	0.1	0.9	0.9	2.1	1.2	0.6	*0*

MC, Mast cells; *Ba*, basophils; *Eo*, eosinophils; *Ne*, neutrophils; *Pl*, platelets; *Fb*, fibroblasts; *TC*, tissue cells; *R*, receptor.

*Cell-type selectivity was obtained by comparing the normalized AD levels of each gene in mast cells, basophils, eosinophils, neutrophils, platelets, CD4+ cells, CD8+ cells, CD14+ cells, CD19+ cells, and fibroblasts.

†The expression of each gene by various tissue cells was determined by searching http://www.lsbm.org/index_e.html. The italicized numeral indicates the number of tissues that have a raw AD level of greater than 200.



Mast cells expressed low levels of FcεRIα compared with basophils, and even neutrophils expressed a substantial level of the receptor (Table II). This raises the possibility that the GeneChip assay might not be suitable for detecting selective molecules. In the present study, however, only the GeneChip data obtained by using cord blood–derived mast cells and lung mast cells could be used because of the strict data selection based on the RNA quality (see the "Methods" section). As has been reported,[14,33] peripheral blood–derived cultured mast cells or skin-derived mast cells expressed approximately 10-fold FcεRIα mRNA compared with that produced by cord blood–derived mast cells (data not shown). Also, as shown in Online Repository Table E1, only 2 of 4 neutrophil samples expressed FcεRIα mRNA. This might be explained by the observation that only neutrophils obtained from some allergic donors express the molecule.[34]

We unexpectedly found 17 granulocyte-selective transcripts, including HTm4. Basophil-selective transcripts, eosinophil-selective transcripts, or both identified in our study could be potential therapeutic targets for allergic diseases because these granulocytes play a crucial role in allergic inflammation.[1,2] Granulocyte-selective transcripts could also be drug targets for other inflammatory diseases, such as systemic vasculitis.[3,4] Analysis of cell type–selective transcripts from database searches is expected to minimize the efforts required for drug discovery. The public database (http://www.lsbm.org/index_e.html) shows that some granulocyte-selective transcripts (18 of 51) detected in our study are abundantly expressed by multiple (>3) organ tissue cell types by using the same GeneChip U133A probe array. Thus the safety of any candidate drug must be evaluated by comparing its efficacy (on granulocytes) with its toxicity (to organs). Six of the 17 novel granulocyte-selective molecules could be excluded from drug development because of their expression in multiple organs unrelated to the diseases. Thus our approach has identified 11 receptors and ion channels with therapeutic potential. Among the 11 receptors and ion channels, 7 were basophil selective, eosinophil selective, or both and were not expressed by other organs, indicating that they might be potential targets for antiallergic drugs.

Finally, it should be stressed that basophils, the rarest leukocytes, have recently been found to play a more crucial role than we ever proposed in the pathogenesis of intractable allergic diseases, such as fatal asthma.[35,36] Thus targeting basophil receptors and ion channels, such as HTm4 and the Ca^{2+} channel *CACNA1D*, is particularly expected for future drug discovery. The importance of molecules known to be expressed by basophils might be reevaluated regarding its selectivity. Freshly isolated resting basophils expressed the highest level of IL-4 compared with other cell types. Because the basophil purification procedure requires more isolation steps, ex vivo manipulation might activate the cells. However, it should also be noted that basophils have been recently found to be the major source of IL-4, at least in asthma models.[37,38]

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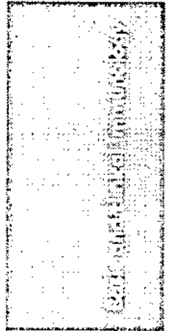

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Effects of lysed *Enterococcus faecalis* FK-23 on allergen-induced serum antibody responses and active cutaneous anaphylaxis in mice

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Summary

Background Our previous studies have presented evidence that lysed *Enterococcus faecalis* FK-23 (LFK), a lysozyme and heat-treated probiotic product, can inhibit allergen-induced local accumulation of eosinophils in mice.

Objective The purpose of this experimental study was to evaluate the influence of orally administrated LFK on the host immune responses.

Methods BALB/c mice were sensitized subcutaneously, and challenged intraperitoneally by cedar pollen allergen. Blood and spleen samples were collected after oral administration of LFK 60 mg/day for 21 days. The serum levels of total and allergen-specific IgE and IgG2a antibodies and the production of IL-4, IL-5 and IFN-γ generated by allergen-stimulated cultured splenocytes were determined. Additionally, the effect of LFK on active cutaneous anaphylaxis (ACA) induced by ovalbumin (OVA) challenge in mice was measured after 28 days LFK treatment.

Results No significant differences in serum immunoglobulin levels, as well as in cytokine production of splenocytes were observed between LFK-treated and control mice ($P > 0.05$). There was, however, an increasing tendency of allergen-specific IgG2a level in mice after LFK treatment for 21 days compared with controls ($P = 0.060$). Furthermore, the serum ratio of specific IgE to IgG2a was found to be significantly decreased in the LFK group ($P = 0.005$). In addition, a significant inhibition of OVA-induced ACA reaction was observed in mice that had been fed for 28 days with LFK compared with control mice ($P = 0.008$).

Conclusion These results suggest that LFK shows an anti-inflammatory effect, which may be part of the mechanism for protection against IgE-mediated allergy.

Keywords allergy, cutaneous anaphylaxis, cytokines, *Enterococcus faecalis*, immunoglobulins, lactic acid bacteria

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Introduction

Intestinal microflora, which plays a crucial role in the programming and development of systemic immunity in early life [1], has received more attention during the last few years as a potential determiner in the development of T-helper type 2 (Th2)-mediated allergic diseases [2]. On the basis of epidemiological data, direct or indirect evidence has shown that alterations of microflora in the gut in childhood are linked to risk of developing atopic sensitization and allergic diseases [3–7]. Moreover, recent clinical trials have demonstrated the beneficial effect of probiotic supplementations made from lactic acid bacteria (LAB) in allergy prevention and therapy [8–13].

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Our previous studies have found that oral administration of lysed *Enterococcus faecalis* FK-23 (LFK), an LAB preparation, can inhibit allergen-induced local accumulation of eosinophils in mice [14], and reduce the number of peripheral blood eosinophils and improve tuberculin responses in patients with allergic rhinitis [15]. It is likely that LFK tends to have an effect in preventing and/or shifting the Th2-dominated immune responses; however, the mechanisms of LFK in combating allergy still need clarification. To address this question, we performed this experimental study and evaluated the influence of orally delivered LFK on the host immune responses.

Methods

Experimental animals

Female BALB/c mice were purchased from Charles River Japan Inc. (Shizuoka, Japan). Five-week-old mice were used

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for the experiments. They were fed on pellet diet CE-2 (Clea Inc., Tokyo, Japan) and received tap water which had been filtrated through a PF filter (Organo Inc., Tokyo, Japan). All animals were housed in cages with a 12 h light/dark cycle. Temperature and humidity were controlled at 25.0 ± 1.0 °C and 55.0 ± 5.0%, respectively.

Preparation of lysed Enterococcus faecalis *FK-23*

LFK, which is a product of lysozyme and heat-treated *E. faecalis* FK-23 strain, was prepared as described previously [14].

Allergen sensitization of BALB/c mice

For clarification of the effect of LFK on allergic responses, a mouse model (Fig. 1a) was established as described previously [14]. In brief, BALB/c mice were sensitized with the purified allergen extract (6 mg/mL) from Japanese cedar (*Cryptomeria japonica*) pollen [16]. 0.1 mL of the extract was injected subcutaneously on days 1 and 2, and then 0.2 mL was injected subcutaneously on days 7, 9 and 15. The mice were challenged on day 21 by the intraperitoneal injection of 0.2 mL cedar pollen allergen extract. 60 mg (0.5 mL) of LFK was orally administrated to the experimental mice ($n = 8$) every day during 21 days of the sensitization period. Saline was fed at a dose of 0.5 mL/day to control mice ($n = 8$) for the same duration.

Measurement of immunoglobulins in sera

Following intraperitoneal challenge by cedar pollen allergen, blood samples of mice were collected at day 22 (Fig. 1a). Serum total and allergen-specific IgE and IgG2a were measured by a sandwich ELISA, as described elsewhere [17, 18] with slight modifications. For measurement of total IgE and IgG2a, 96-well plates for ELISA were coated with each isotype specific anti-mouse immunoglobulin antibody (PharMingen, San Diego, CA, USA) by incubation overnight at 4 °C, and then further treated with 10% fetal calf serum (FCS) added in phosphate-buffered saline (PBS) for 4 h at room temperature to block any nonspecific binding. Subsequently, the serial dilutions of mouse serum samples were incubated in the well for 1 h at room temperature. Purified mouse IgE (PharMingen) and IgG2a (Ancell Corporation, Bayport, MN, USA) antibodies were used as the standard, respectively. After being washed three times PBS containing 0.05% Tween 20, biotin-conjugated rat anti-mouse IgE (PharMingen) and peroxidase-conjugated rabbit anti-mouse IgG2a (ICN Biomedicals, Aurora, OH, USA) antibodies were added to the wells, followed by the addition of streptoavidin-peroxidase (Zymed Laboratories, San Francisco, CA, USA) for 1 h at room temperature. After washing six times, the plates were developed using a tetramethylbenzidine (TMB) substrate solution. The reactions were terminated by the addition of 1 N phosphoric acid. The plates were read in a microplate reader, MTP-300 (Corona Inc., Ibaragi, Japan) at 450 nm, and the concentration of each antibody was calculated according to the individual standard curve.

For determination of the serum levels of allergen-specific IgE and IgG2a, samples with the 2^{-2} and 2^{-4} dilutions were placed on ELISA plates coated previously with cedar pollen allergen. The experimental procedure then proceeded with the methods as described above, and the antibody levels were expressed as the absorbance at 450 nm.

Measurement of cytokine production by cultured splenocytes

Mouse spleens were removed aseptically at day 22 (Fig. 1a), and a single-cell suspension was prepared by gently squeezing them between two glass slides in PBS. Splenocytes were washed and suspended in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan). 150 μL of cultures (5×10^5 cells/mL) were prepared in 96-well, flat-bottom plates. Cultured splenocytes were then stimulated with 50 μL extract of cedar pollen allergen for 48 h at 37 °C in a humidified CO_2 incubator. The levels of IL-4, IL-5 and IFN-γ in harvested culture supernatants were determined by an ELISA kit (BioSource International, Camarillo, CA, USA) in accordance with the manufacture's recommendations.

Active cutaneous anaphylaxis

For determination of the influence of LFK on active cutaneous anaphylaxis (ACA), a different mouse model

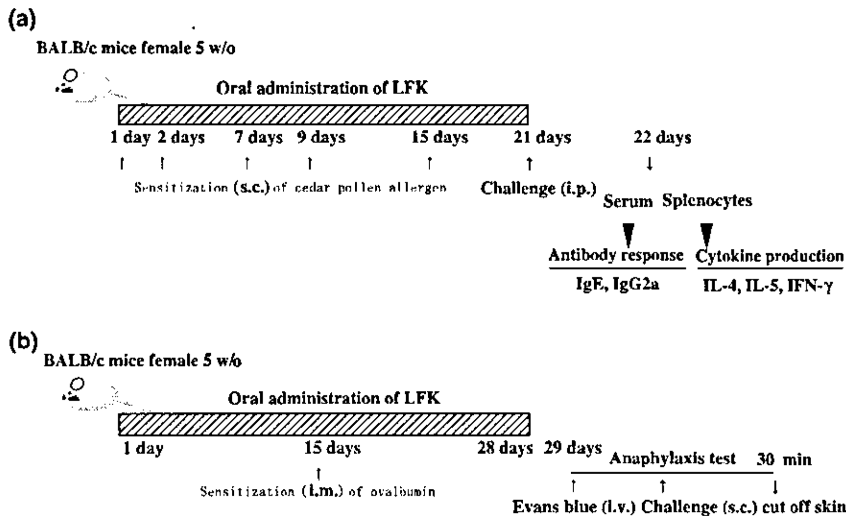

Fig. 1. Experimental procedures in this study. BALB/c mouse models established for testing the effects of lysed *Enterococcus faecalis* FK-23 (LFK) on the serum antibody responses and cytokine production by splenocytes (a), and on the active cutaneous anaphylaxis reaction (b), respectively. s.c., subcutaneous; i.p., intraperitoneal; i.m., intramuscular; i.v., intravenous.

(Fig. 1b) was established as follows. 60 mg (0.5 mL) of LFK was orally administrated to 5-week-old female BALB/c mice ($n = 15$) per day for 28 days. Saline was fed to the age- and sex-matched mice ($n = 15$) at a dose of 0.5 mL every day for the same duration as the control. The experimental mice were immunized intramuscularly with 0.1 mL of ovalbumin (OVA; Sigma Chemical, St Louis, MO, USA) saline solution (3 mg/mL) at day 15. Following an intravenous injection of 0.05 mL of 1% Evans Blue (Sigma Chemical), ACA was elicited in the skin of belly by subcutaneous injection of 0.05 mL of OVA at day 29. Thirty minutes after OVA challenge, mice were killed and a piece of skin shaved from a mouse was placed in 3 mL of mixture solution containing 0.05% sodium sulphate anhydrous and 99.5% acetone (Wako Pure Chemical, Tokyo, Japan). After vigorous shaking, the absorbance of extravasated dye was measured colorimetrically at 620 nm.

Statistical analysis

Data were presented as mean ± SEM. The SPSS 10.0J (SPSS Inc., Chicago, IL, USA) was used for analysis. Statistical differences between the groups were determined by unpaired Student's *t*-test. Total serum IgE and IgG2a levels were logarithm-transformed to normalize the distribution. Differences were considered significant if the *P*-value for the effect was less than 0.05.

Results

Effect of lysed Enterococcus faecalis *FK-23 on serum immunoglobulin E and immunoglobulin G2a antibodies*

Total and allergen-specific IgE and IgG2a antibodies in sera from the mice that had been fed with LFK for 21 days and the control mice were measured. As shown in Table 1, no significant differences in the serum levels of total as well as allergen-specific IgE and IgG2a were found between two groups ($P > 0.05$), but the specific IgG2a level (absorbance at 450 nm) was observed to be an increasing tendency in LFK-treated mice (0.32 ± 0.03) than that in controls (0.26 ± 0.01, $P = 0.060$). Interestingly, the ratio of specific IgE to IgG2a was significantly lower in the LFK group (0.68 ± 0.08) than that in the control group (1.09 ± 0.09, $P = 0.005$).

Effect of lysed Enterococcus faecalis *FK-23 on interleukin-4, interleukin-5 and interferon-γ production by splenocytes*

To further verify the effect of LFK on cytokine production generated by allergen-stimulated cultured splenocytes, IL-4, IL-5 and IFN-γ from the culture supernatants were examined. As shown in Table 2, no significant difference in the production of IL-4, IL-5 or IFN-γ could be found between LFK-treated and control mice ($P > 0.05$). Also, no significant difference was observed in the ratio of IL-4 or IL-5 to IFN-γ between experimental groups ($P > 0.05$).

Effect of lysed Enterococcus faecalis *FK-23 on active cutaneous anaphylaxis*

The reaction of ACA following OVA challenge was compared in control mice and in mice that had been orally administrated with LFK for 28 days. As shown in Table 3, OVA-induced vascular permeability (absorbance at 620 nm) was significantly lower in LFK-treated mice (0.33 ± 0.04) than that in controls (0.50 ± 0.05, $P = 0.008$).

Table 1. Effect of LFK on serum IgE and IgG2a antibodies

	Control ($n = 8$)	LFK-treated ($n = 8$)	*P*-value
Total IgE (log ng/mL)	3.43 ± 0.09	3.25 ± 0.07	0.133
Total IgG2a (log ng/mL)	5.92 ± 0.07	5.82 ± 0.04	0.228
Total IgE/IgG2a ratio	0.58 ± 0.02	0.56 ± 0.01	0.335
Specific IgE (absorbance at 450 nm)	0.28 ± 0.03	0.22 ± 0.03	0.165
Specific IgG2a (absorbance at 450 nm)	0.26 ± 0.01	0.32 ± 0.03	0.060
Specific IgE/IgG2a ratio	1.09 ± 0.09	0.68 ± 0.08	0.005

Values are presented as mean ± SEM.
LFK, lysed *Enterococcus faecalis* FK-23.

Table 2. Effect of LFK on IL-4, IL-5 and IFN-γ production by splenocytes

	Control ($n = 8$)	LFK-treated ($n = 8$)	*P*-value
IL-4 (pg/mL)	21.87 ± 2.87	22.30 ± 3.38	0.924
IL-5 (pg/mL)	242.83 ± 55.10	270.30 ± 57.11	0.736
IFN-γ (pg/mL)	10.02 ± 5.46	18.54 ± 3.22	0.235
IL-4/IFN-γ ratio	9.56 ± 4.22	0.93 ± 0.20	0.096*
IL-5/IFN-γ ratio	126.53 ± 60.72	14.30 ± 6.90	0.124*

Values are presented as mean ± SEM.
*t-test.
LFK, lysed *Enterococcus faecalis* FK-23.

Table 3. Effect of LFK on active cutaneous anaphylaxis (ACA)

	Control ($n = 15$)	LFK-treated ($n = 15$)	*P*-value
ACA (absorbance at 620 nm)	0.50 ± 0.05	0.33 ± 0.04	0.008

Values are presented as mean ± SEM.
LFK, lysed *Enterococcus faecalis* FK-23.

Discussion

Recent clinical trials have highlighted a role of probiotic therapy in the prevention or management of allergic diseases, especially atopic dermatitis/eczema [8–12], indicating the competence of probiotics in anti-allergy immunoregulation. The term probiotics referrs to preparations of microorganisms or components of microbial cells that have a beneficial effect on the health and well-being of the host when ingested [19, 20]. The most commonly used probiotics are *Lactobacillus* and *Bifidobacterium* species, but *Enterococcus* strains and *Escherichia coli* have also been proposed as such in the light of the above definition [20].

LFK is a product of *E. faecalis* FK-23 strain components without viable cells. Using a BALB/c mouse model in this study, we found no significant differences in the serum levels of total as well as allergen-specific IgE and IgG2a antibodies

between the mice that had been fed with LFK for 3 weeks and the control mice. However, after LFK treatment an increasing tendency of specific IgG2a level was shown ($P = 0.060$), and with the results we found significantly reducing specific IgE/IgG2a ratio in LFK-treated mice compared with controls ($P = 0.005$). Given that the serum ratio of IgE to IgG2a is used as a surrogate marker to monitor the Th1 (T-helper type 1)/Th2 balance, our data suggested that oral administration of LFK might have an effect on immune balance between Th1 and Th2 immunities. This hypothesis is supported by Sudo et al. [18] showing that an antibiotic-induced Th2-skewed response in mice was reversed by supplementation with a living strain of *E. faecalis*. In addition, experimental studies have indicated that after oral feeding of heat-killed *Lactobacillus casei* strain, the antigen-specific IgE and Th2 cytokine-dependent inflammatory responses were inhibited [21, 22]. It is possible that probiotic LAB may change the imbalance between Th1 and Th2 responses through down-regulation of T cell activation and IL-4-induced IgE synthesis by indirect mechanisms as suggested by Pessi et al. [23, 24].

The Th1/Th2 decision is crucial to effective immunity and it is likely that many interlocking factors (e.g. antigen-presenting cells and cytokines) contribute to its decision [25]. It is known that the Th2-type cytokines such as IL-4 and IL-5 are essentially associated with IgE-mediated allergy because of their actions on some specific cells known to be involved in the allergic inflammatory reactions. In contrast, the Th1-type cytokine, IFN-γ, has an inhibitory effect on IgE and IgG1 secretion, whereas an enhancing effect on IgG2a secretion *in vivo*. It has been reported that heat-killed *Lactobacillus* strains can increase *in vitro* production of IFN-γ, but reduce the levels of allergen-driven IL-4 and IL-5 either in mouse models [21, 22] or in allergic patients [26], suggesting that some strains of LAB may exhibit an anti-Th2 activity. Conversely, a recent clinical study by Rosenfeldt et al. [12] found no significant changes in the production of either Th1 or Th2 cytokines (IFN-γ, IL-2, IL-4 and IL-10) from peripheral blood mononuclear cells after 6 weeks' administration of probiotic *Lactobacillus* strains to children with atopic dermatitis, but a decrease of serum eosinophil cationic protein levels was observed. In the present study, we also found no significant difference in the production of IL-4, IL-5 or IFN-γ generated by allergen-stimulated cultured splenocytes between the mice orally applied for 3 weeks with LFK and the control mice. Further clarification is needed on whether a longer period of administration of probiotic LAB is required to produce remarkable changes in the cytokine profiles.

Interestingly, in this study we could show that BALB/c mice fed with LFK for 4 weeks effectively reduced the ACA reaction induced by OVA challenge compared with controls ($P = 0.008$). Our finding, together with the published data [27] that certain *Lactobacillus* strain can diminish systemic anaphylaxis in a food allergy mouse model, suggested a possible inhibitory activity of probiotic LAB on IgE (perhaps including IgG1)-mediated mast cell degranulation that drives systemic or topical allergic reactions.

In summary, according to the results presented here and our previous observation that oral LFK can inhibit allergen-induced local eosinophilia in mice [14], we speculate that the anti-inflammatory effects of LFK may be part of the mechanism in limiting IgE-mediated allergy. It also prompts further investigation of other mechanisms involved and the beneficial effect of LFK on allergic diseases *in vivo*.

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Polymorphisms in *ADAM33* are associated with allergic rhinitis due to Japanese cedar pollen

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Summary

Background A recent report provided evidence that a disintegrin and metalloprotease domain 33 (*ADAM33*), a member of the ADAM family, is a novel susceptibility gene in asthma linked to bronchial hyper-responsiveness. However, there has been no investigation of the genetic role of *ADAM33* variants in nasal allergy.

Objective The purpose of this study was to test the association between *ADAM33* polymorphisms and Japanese cedar pollinosis (JCPsis), a most common seasonal allergic rhinitis in Japan.

Methods We conducted a case–control association study among a Japanese population, involving 95 adult individuals with JCPsis and 95 normal healthy controls. A total of 22 single-nucleotide polymorphisms (SNPs) in *ADAM33* were genotyped using PCR-based molecular methods.

Results Six SNPs of *ADAM33* gene, three in introns (7575G/A, 9073G/A and 12540C/T) and three in the coding region (10918G/C, 12433T/C and 12462C/T), were strongly associated with JCPsis ($P = 0.0002 - 0.022$ for absolute allele frequencies) and most of the SNPs were in linkage disequilibrium with each other. A higher frequency of the common alleles of these SNPs was noted for the subjects with JCPsis in comparison with healthy controls. We also identified a haplotype associated with the disease susceptibility. In addition, associations were found between *ADAM33* polymorphisms and various cedar pollinosis phenotypes including clinical severity, eosinophil counts in nasal secretion and allergen-specific IgE levels in sera, but not total serum IgE levels.

Conclusion These results indicate that polymorphisms in the *ADAM33* gene are associated with susceptibility to allergic rhinitis due to Japanese cedar pollen, but the functional relationship still needs clarification.

Keywords *ADAM33*, allergic rhinitis, Japanese cedar pollinosis, single-nucleotide polymorphism, susceptibility gene

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Introduction

Japanese cedar pollinosis (JCPsis) is an IgE-mediated type I allergy affecting the nasal mucosa caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen (JCP). It is one of the most common allergic diseases in Japan and represents a public health issue affecting 5–20% of the Japanese population, with increasing prevalence over the past two to three decades [1–3]. JCPsis is known to be a complex disease with genetic predisposition [4], although the underlying genes have not been pinpointed. Several previous studies, focused on the relationship between HLA class II molecules and JCPsis, showed antigen-specific T cell responsiveness to the major allergens of JCP was strikingly associated with certain specific *HLA-D* alleles [5–7]. Recently, a significant association has been found between a coding variant (Glu237Gly) of the β chain of the high-affinity IgE receptor (FcεRIβ) and JCPsis, as well as serum total and specific IgE levels [8], suggesting a strong genetic component to the onset and disease phenotype of JCPsis.

Allergic rhinitis (AR) and asthma may both be manifestations of the atopic syndrome, affecting different anatomical parts of the respiratory tract, which share a common genetic background [9], and are characterized by a similar inflammatory process [10, 11]. Significant progress has been made in

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the study of genetics of atopy during the past decade since Cookson et al. [12], in 1989, first reported a linkage between generalized IgE responsiveness and a variable number of tandem repeats polymorphism on chromosome 11q13 in British families. Nevertheless, intensive research on the complex genetics of allergic disease has focused primarily on asthma and related traits, with very little attention given to rhinitis [9, 13]. Recent information regarding asthma genetics comes from the work of Van Eerdewegh et al. [14]. These investigators performed a genetic study by combining linkage and association approaches in Caucasian affected sib-pair families from the UK and the US, and identified a disintegrin and metalloprotease domain 33 (*ADAM33*) as a major susceptibility gene for asthma linked to bronchial hyper-responsiveness (BHR). Most recently, the association of *ADAM33* polymorphisms with asthma and related phenotypes has been replicated in four unique asthma populations (African American, US white, US Hispanic and Dutch white) [15] and in the German samples [16], but not in the Latino populations (Puerto Rican and Mexican) [17].

We hypothesized that association of the *ADAM33* gene with asthma may not be specific for asthma *per se* but rather may reflect an overall genetic predisposition for allergic airway diseases. To clarify whether the *ADAM33* polymorphisms relate to JCPsis, a most important springtime AR in Japan, we genotyped and analysed a total of 22 single-nucleotide polymorphisms (SNPs) within the *ADAM33* locus in a case–control association study.

Materials and methods

Subjects

The study included 95 unrelated adult individuals with JCPsis and 95 age-matched unrelated healthy controls. All subjects were from the population of Kinki area, west Japan. The diagnosis of JCPsis was based on a positive history of rhinitis symptoms during the cedar pollen season, and positive allergen-specific IgE reactivity to JCP in serum. Exclusion criteria were a present or past history of asthma and asthma-like symptoms. Among a total of 95 patients with JCPsis, 45.3% were also allergic to house dust mites (HDM), and 18.9% of patients had perennial symptoms with seasonal exacerbations. None of the patients had received specific immunotherapy for allergic conditions. The control subjects were all symptom free, had no history of atopic disorders, and had negative allergen-specific IgE against JCP as well as HDM. All participants gave written informed consent to participate in the study, according to the process approved by the Ethical Committee in RIKEN Yokohama Institute.

Clinical parameters

Detailed characteristics of recruited patients are described in Table 1. Among a total of 95 patients with JCPsis, five cases (5.3%) were mild, 22 cases (23.2%) were moderate and 68 cases (71.6%) were diagnosed as severe according to the scores of three main nasal symptoms (sneezing, rhinorrhoea and nasal obstruction), on the basis of the clinical severity classification for AR (Okuda's method) described in detail elsewhere [18, 19].

Table 1. Phenotypic characteristics of recruited patients

Number of subjects	95
Clinical severity	
Mild	5 (5.3%)
Moderate	22 (23.2%)
Severe	68 (71.6%)
Total serum IgE (IU/mL)	
Geometric mean	162.5
Ranges	5.3–10 000
RAST scores for Japanese cedar pollen	
Score 2	9 (9.5%)
Score 3	42 (44.2%)
Score 4	25 (26.3%)
Score 5	16 (16.8%)
Score 6	3 (3.2%)
RAST scores for house dust mites	
Score ≥2	43 (45.3%)
Eosinophil counts in nasal secretions	
None	13 (13.7%)
1+	36 (37.9%)
2+	16 (16.8%)
3+	30 (31.6%)

Serum total and allergen-specific IgE levels were measured for all participants with an AutoCAP System (Pharmacia Diagnostics AB, Uppsala, Sweden). The geometric mean (range) of total IgE levels in patients and control subjects was 162.5 (5.3–10000) IU/mL and 30.0 (3.2–240) IU/mL, respectively. Subjects with JCPsis had higher total IgE levels than healthy controls (2.21 ± 0.51 (mean ± SD) log IU/mL vs. 1.48 ± 0.51, $P<0.001$, *t*-test). Allergen-specific IgE levels were determined by RAST. The grading of RAST scores 0–6 was made according to the manufacturer's instructions, with a specific IgE level of 0.7 UA/mL (RAST score 2 or more) being positive. In addition, to evaluate the nasal eosinophilia in patients with JCPsis, eosinophils in nasal secretion (nEOS) obtained from nose blowing were stained by Hansel staining and semi-quantitated ranging from 0 to 3+ (0: none; 1+: scattered; 2+: between 1+ and 3+; and 3+: clustered) [18, 19].

SNPs

We screened a total of 22 SNPs at the human *ADAM33* locus as the target polymorphisms for a case–control association study. The SNPs studied were based on the database of SNPs in Japanese population (JSNP, data are available through website at http://snp.ims.u-tokyo.ac.jp/) [20, 21], and our own data for SNP discovery (data not shown). The 22 SNPs are shown in Table 2, five SNPs (−3989T/C, −3982C/T, −3835C/T, −3705C/A and −85T/C) are in the 5′ genomic region, 10 SNPs (6716G/C, 7575G/A, 7667A/G, 9073G/A, 11188A/T, 11434C/A, 12540C/T, 12601T/G, 12946G/A and 12954C/T) are in introns, four SNPs (7441A/G, 10918G/C, 12433T/C and 12462C/T) are in the coding region and the remaining three SNPs (13236T/C, 13506C/G and 13527A/G) belong to the 3′ untranslated region (3′UTR) of exon 22.