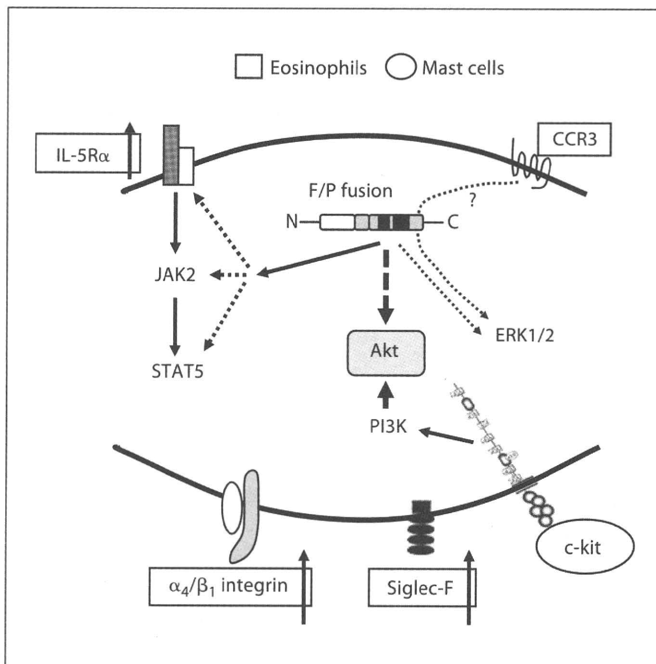

Fig. 1. Intracellular signaling of FIP1L1/PDGFRα+ (F/P) cells. FIP1L1/PDGFRα+ (F/P) primary mouse eosinophils express up-regulated IL-5Rα and FIP1L1/PDGFRα activates the JAK2/STAT5 pathway. The CCR3/ERK1/2 signaling pathway may be amplified by FIP1L1/PDGFRα expression [25]. Up-regulated expressions of α_4 integrin and Siglec-F were observed in FIP1L1/PDGFRα+ murine eosinophils [8]. FIP1L1/PDGFRα synergizes with SCF stimulation via c-kit to activate Akt signaling in mouse mast cells. Eosinophils and mast cells also express c-kit and IL-5Rα, respectively.

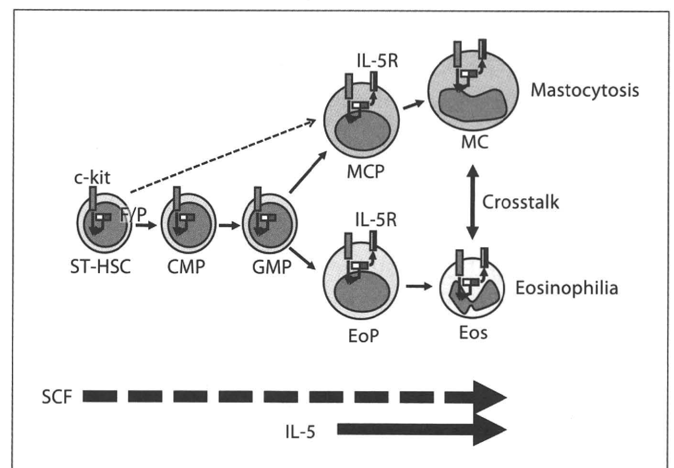

Fig. 2. FIP1L1/PDGFRα in conjunction with SCF and IL-5 promote leukemic hematopoiesis and eosinophil and mast cell (MC) development. FIP1L1/PDGFRα may occur in hematopoietic stem cells or early progenitor cells resulting in the expression of FIP1L1/PDGFRα in most hematopoietic cells. Progenitors including earlier and mature eosinophils and MCs express c-kit. In contrast, IL-5Rα expression has been observed on eosinophil progenitor (EoP), MC progenitor (MCP) and mature eosinophils and MCs. FIP1L1/PDGFRα enhances SCF/c-kit signaling by sharing downstream signaling and up-regulates IL-5Rα expression facilitating its intracellular signaling. There is significant crosstalk between eosinophils and MCs. These findings imply that FIP1L1/PDGFRα in collaboration with SCF may affect leukemic myeloproliferation, and synergistically with IL-5 expand and activate MC and eosinophil lineages. ST-HSC = Short-term HSC; CMP = common myeloid progenitor; GMP = granulocyte-macrophage progenitor.

pendent arises. In fact, following blockade of the the c-kit signaling pathway using anti-c-kit antibody, tissue mast cells and circulating levels of MMCP-1 were significantly decreased, suggesting that tissue infiltration of FIP1L1/PDGFRα fusion-positive mast cells are associated with SCF/c-kit signaling. The ex vivo cytokine-dependent mast cell differentiation of FIP1L1/PDGFRα-expressing HSC/Ps was largely dependent on the use of SCF in the culture conditions. Moreover, FIP1L1/PDGFRα fusion-positive mast cells showed prolonged survival and enhanced migration toward SCF. Specific synergistic stimulation of the Akt signaling pathway by FIP1L1/PDGFRα and SCF indicated collaboration of two tyrosine kinase activities in their downstream signaling pathways. Taken together, FIP1L1/PDGFRα synergizes with the SCF/c-kit pathway to promote mast cell development, activation and survival both in vivo and in vitro [9].

Pathogenesis of FIP1L1/PDGFRα-Positive HES/CEL/SM Associated with SCF and IL-5

Previously, we reported that the induction of murine HES/CEL by FIP1L1/PDGFRα requires a second event that is associated with IL-5 overexpression [8]. In addition, the level of expression of IL-5Rα was exclusively up-regulated in FIP1L1/PDGFRα-positive splenocytes and FIP1L1/PDGFRα fusion protein shares the downstream JAK2/STAT5 pathway with IL-5 signaling (fig. 1) [16]. Interestingly, polymorphisms of the human *IL-5RA* gene have been found linked to the constitutional *IL-5RA* genotype and the severity of FIP1L1/PDGFRα-positive CEL [17]. These findings suggest that amplification of IL-5 signaling by FIP1L1/PDGFRα triggers a CEL-like disease. Interestingly, IL-5Rα is expressed on eosinophil and mast cell progenitors [18, 19] as well as mature eosinophils [20]

and mast cells [21], whereas c-kit expression is not only found on progenitors but also on mature eosinophils and mast cells [14, 18, 19]. Importantly, expression of the *FIP1L1/PDGFRA* fusion gene or deletion of the surrogate marker CHIC2 have been detected in non-eosinophilic cells, including neutrophils, monocytes, mast cells, lymphoid lineage cells and bone marrow CD34-positive cells in part of the patients, suggesting that the fusion of the *FIP1L1/PDGFRA* genes may occur in HSCs or early progenitors [22–24]. Taken together, these findings imply that FIP1L1/PDGFRα in collaboration with SCF may affect leukemic myeloproliferation and synergistically with IL-5 expand and activate mast cell and eosinophil lineages (fig. 2).

Conclusion

HES/CEL has attracted a lot of attention since the patients were successfully treated with imatinib mesylate, and subsequently the target, FIP1L1/PDGFRα, was discovered in a large number of patients initially diagnosed as myeloproliferative variant of HES. To our knowledge, there is little doubt that FIP1L1/PDGFRα preferentially affects eosinophil and mast cell proliferation, survival, differentiation and tissue infiltration, and leukemogenesis is induced combined with systemic or local extrinsic factors, as demonstrated by crucial roles of IL-5 and SCF in the pathogenesis of FIP1L1/PDGFRα-initiated HES/CEL/SM. This disease, an example of the crosstalk between oncogenesis and inflammation, represents an excellent model to study cellular integration of biochemical signals in cancer, being responsible for crucial aspects of cancer biology, e.g. cell proliferation, survival, tissue invasion and communication with the specific tissue microenvironment.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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High frequencies of simultaneous *FLT3*-ITD, *WT1* and *KIT* mutations in hematological malignancies with *NUP98*-fusion genes

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Acute myeloid leukemia (AML) is heterogeneous in clinical features and molecular pathogenesis. Cooperating alterations of several genes, including oncogenes or tumor suppressor genes, lead to AML development.[1] AML leukemogenesis is thought to require at least two different types of genetic change: class I mutations, which confer a proliferative or survival advantage; and class II mutations, which block myeloid differentiation and provide self-renewability.[1] In hematological malignancies with 11p15 translocations, the nucleoporin (*NUP*) *98* gene is reportedly fused to various partner genes, often including homeobox genes, such as *HOXA9, A11, A13, C11, C13, D11, D13* and *PMX1*.[2] With respect to the oncogenic mechanism of NUP98-HOX fusion proteins, a previous study using a murine bone marrow transplantation assay revealed that NUP98-HOXA9, -HOXD13 and -PMX1 fusion proteins induce myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN), which progress to AML.[2] This latency period indicates that additional genetic events might be required for leukemic transformation. Therefore, we examined somatic mutations of the *FLT3, KIT, WT1, RUNX1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes, which are prevalent in AML, in leukemia patients with *NUP98* fusion genes. This study was approved by local ethical committee.

Sixteen patients with chromosomal 11p15 translocations included nine with *NUP98-HOXA9*, two with *NUP98-HOXA13*, two with *NUP98-HOXA11* and one each with *NUP98-HOXC11*, *NUP98-HOXD11*, *NUP98-HOXD13* or *NUP98-NSD3* (Table 1). The partner gene fused to *NUP98* could not be detected in one patient with t(4;11)(q21;p15); however, fluorescent *in situ* hybridization analysis using a probe containing *NUP98* showed split signals (data not shown). No patients had any additional chromosomal abnormality except for chromosomal 11p15 translocations (Supplementary data). Two patients with t(7;11)(p15;p15) had double *NUP98* fusion transcripts: patient (PN) 13 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA13* fusions, and PN14 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA11* fusions. In all, 15 of the 16 patients with *NUP98*-related hematological malignancies were diagnosed as having myeloid malignancies, and the other patient (PN16) were initially diagnosed as having T-cell non-Hodgkin's lymphoma with t(4;11)(q21;p15), and transformed into acute myelomonocytic leukemia with the same t(4;11) (lineage switch). Patients with myeloid malignancies consisted of 10 patients with AML, 2 patients with MDS and 3 patients with MPN.

We examined the internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations of the *FLT3* gene in 16 patients, and detected ITDs in nine (56.3%) patients, and TKD mutations in none (Table 1, Figure 1a). The incidence of *FLT3*-ITD in our study was much higher than that in an AML cohort reported previously (12–35%).[1] A high frequency of *FLT3*-ITD was previously reported in 30–35% of AML patients with either normal karyotype or with t(15;17)(q21;q11) resulting in *PML-RARA*, and in 70% of AML patients with t(6;9)(p23;q34) resulting in *DEK-CAN/NUP214*.[1] Interestingly, both *NUP98* and *NUP214* encode a part of the nucleoporin complex. The general activation effects on reporters of the DEK-CAN/NUP214 fusion protein are specific for myeloid cells.[3] Moreover, in murine bone marrow transplantation assays, NUP98-related fusion proteins such as NUP98-HOXA9, -HOXD13 and -PMX1 induced MDS or MPN, which progressed to AML.[2] These results demonstrate that the nucleoporin-related proteins share a common ability for myeloid differentiation. Furthermore, the very tight correlation between *nucleoporin*-related fusion genes and *FLT3*-ITD suggest that *FLT3*-ITD may contribute to the myeloid leukemogenesis involved in *nucleoporin*-related fusions.

We further examined mutations of the *KIT, WT1, AML1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes,[4] which are prevalent in AML. *KIT, NRAS* and *KRAS* mutations were found in four (25.0%), three (18.8%) and two (12.5%) patients, respectively (Table 1, Figure 1b). *WT1* aberrations were found in eight patients (50.0%; Table 1, Figure 1c). No mutations were found in the other four genes (*RUNX1, CEBPA, NPM1* and *MLL*). The mutations in *KIT* were all missense mutations including Val399Ile, Met541Leu and Asp816Val, and all mutations of *NRAS* and *KRAS* were Gly13Asp. All of *KIT, NRAS* and *KRAS* mutations were heterozygous. The aberrations in *WT1* comprised a frameshift insertion of exon 7 in four patients, missense mutation of exon 9 in one, deletion of exon 5 in one and deletion of the whole cording region in two. Frameshift and

Table 1 Clinical features and additional mutations of patients with *NUP98*-related leukemias

PN	*Age*	*Sex*	*Disease*	*WBC at diagnosis*	*Karyotype*	*Fusion partner gene of NUP98*	*CR*	*Relapse*	*Therapy*	*Prognosis*	*FLT3*	*KIT*	*WT1*	*NRAS*	*KRAS*
PN1	14	M	AML-M1	12 500	t(11;12)	*HOXC11*	yes	yes	Chemo+SCT	Death	ITD	Val399Ile	del	WT	WT
PN2	12	F	AML-M2	133 100	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT
PN3	13	M	AML-M2	460 000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN4	13	F	AML-M2	147 000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Alive	WT	WT	WT	WT	WT
PN5	15	M	AML-M2	22 700	t(7;11)	*HOXA9*	yes	no	Chemo+SCT	Alive	WT	WT	WT	WT	Gly13Asp
PN6	57	M	AML-M2	252 000	t(7;11)	*HOXA13*	yes	yes	Chemo	Death	ITD	WT	WT	WT	WT
PN7	38	M	AML-M2	6400	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Asp816Val	ins4bpfsX	WT	WT
PN8	15	M	AML-M4	187 900	t(2;11)	*HOXD11*	yes	no	Chemo+SCT	Alive	WT	WT	ins4bpfsX	WT	Gly13Asp
PN9	56	M	AML-M4	204 500	t(7;11)	*HOXA9*	yes	yes	Chemo	Lost to follow-up	ITD	WT	WT	WT	WT
PN10	62	M	AML-M4	6500	t(2;11)	*HOXD13*	yes	no	Chemo	Alive	ITD	WT	WT	WT	WT
PN11	60	M	RA	6250	t(8;11)	*NSD3*	no	ND	Chemo	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN12	69	F	RAEB	2500	t(7;11)	*HOXA9*	no	ND	Chemo	Death	WT	WT	WT	WT	WT
PN13	45	M	CMML	29 800	t(7;11)	*HOXA9/HOXA13*	yes	yes	Chemo	Death	ITD	WT	Arg250Trp	WT	WT
PN14	58	F	CML(Ph-)	11 200	t(7;11)	*HOXA9/HOXA11*	yes	no	Chemo	Alive	ITD	WT	del	WT	WT
PN15	3	F	JMML	39 400	t(7;11)	*HOXA11*	yes	no	Chemo+SCT	Alive	WT	WT	del exon5	Gly13Asp	WT
PN16	51	F	T-NHL	2600	t(4;11)	undetermined	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT

Abbreviations: AML, acute myeloid leukemia; Chemo, chemotherapy; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CR, complete remission; del, deletion; F, female; JMML, Juvenile myelomonocystic leukemia; M, male; ND, not determined; Ph-, Philadelphia chromosome; PN, patient number; RA, refractory anemia; RAEB-t, refractory anemia with excess of blasts in transformation; SCT, stem cell transplantation; T-NHL, T-cell non-Hodgkin's lymphoma; WBC, white blood cell; WT, wild type.
t(11;12), t(11;12)(p15;q13); t(2;11), t(2;11)(q31;p15); t(4;11), t(4;11)(q21;p15); t(7;11), t(7;11)(p15;p15); t(8;11), t(8;11; p11; p15).

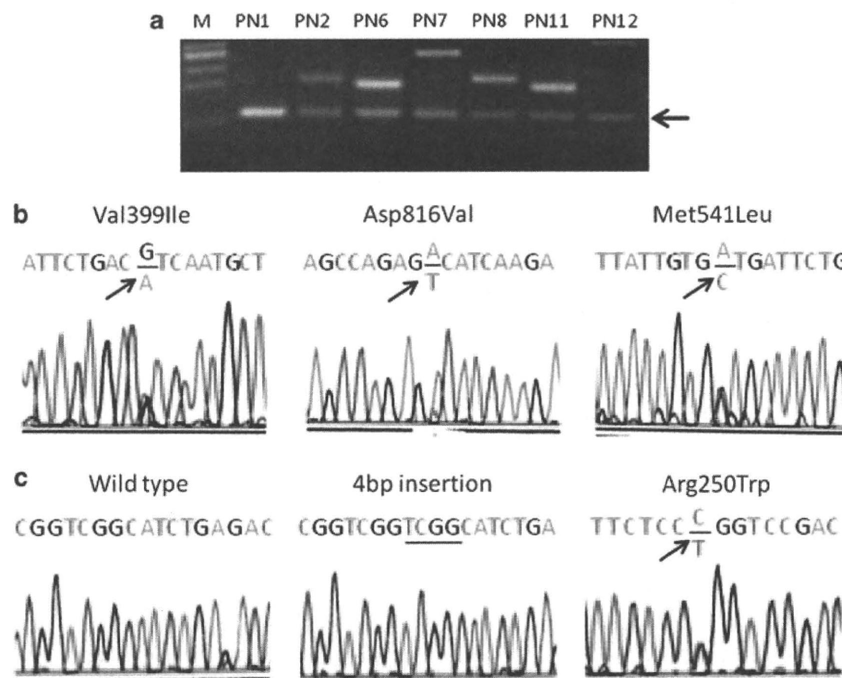

Figure 1 *FLT3*-ITD, *KIT* and *RAS* mutations, and *WT1* aberrations. (**a**) Identification of *FLT3*-ITD by reverse transcription PCR. M, size marker; arrow indicates wild-type allele. (**b**) *KIT* mutations. All figures show the sequence of PCR products. (**c**) *WT1* aberrations. Left panel shows wild type of *WT1* exon 7. Middle panel shows 4-bp insertion in exon 7 of *WT1*. Right panel shows *WT1* missense mutation. Left and middle panels show the sequence of each plasmid subclone, and right panel shows that of PCR products.

missense mutation of *WT1* are heterozygous, whereas deletion was homozygous. *FLT3*-ITD, *KIT* and *RAS* mutations reportedly confer cellular proliferative abilities.[1] In our study, 14 patients (88%) had at least one mutation involved in cellular proliferation (*FLT3, KIT* or *RAS*). Recently, Chou *et al.*[5] reported that the *NUP98-HOXA9* fusion is strongly associated with *KRAS* and *WT1* mutations. *Nras* and *Kras* mutations were frequently found in AML developed in transgenic mice expressing *NUP98-HOXD13*.[6] These results indicate that *NUP98*-related leukemias have a high frequency of mutations involved in growth advantage.

Interestingly, five of the six patients with *WT1* aberrations had *FLT3*-ITD, and three of the five patients with both *FLT3*-ITD and *WT1* aberrations had a *KIT* mutation, although the simultaneous *FLT3*-ITD and *KIT* mutations are reportedly very rare[1]. These results suggest that the *NUP98*-related leukemias share a distinct molecular subgroup in leukemias. In addition, all four patients with *KIT* mutations had both *FLT3*-ITD ($P=0.04$) and *WT1* aberrations ($P=0.03$), whereas all five patients with *RAS* mutations did not have *FLT3*-ITD. In all, 14 (88%) of the 16 patients had either *FLT3*-ITD or *RAS* mutations, but they were mutually exclusive as described in previous papers.[1] These

suggest the distinct molecular basis between *NUP98*-related leukemias having *FLT3*-ITD and those having *RAS* mutations.

The relationships between clinical features and gene mutations were described in Table 1. In our study, male patients were more likely than female patients to have *FLT3*-ITD ($P=0.01$) and patients with *FLT3*-ITD have leukocytosis ($P=0.08$) more than those without *FLT3*-ITD. Patients with *RAS* mutations were significantly younger than those without the mutations (median age of 15 vs 56 years; $P=0.04$). In total, 9 (64.3%) of the 14 patients who achieved complete remission relapsed, and 9 (60.0%) of the 15 patients whose data were available died, although they were treated by different protocols (Table 1). All three patients who had both *FLT3*-ITD and *KIT* mutations, and five (83.3%) of the six patients who had both *FLT3*-ITD and *WT1* aberrations, died. Many studies have shown that *FLT3*-ITD is related to a poor prognosis in AML patients,[1] and that *KIT* mutations are associated with a worse outcome in CBF-leukemia patients.[1] *WT1* mutations are also reported to be a poor prognostic factor in adult AML patients with normal karyotypes.[7] These results suggest that simultaneous occurrence of *FLT3*-ITD, *KIT* mutations and *WT1* aberrations in *NUP98*-related leukemia may be associated with poor prognosis.

FLT3-ITD, *KIT* and *RAS* mutations lead to constitutive activation of downstream pathway, resulting in acquirement of a proliferative advantage.[1] In a mouse model, *FLT3*-ITD alone does not induce AML, and *RAS* mutations can induce myeloid leukemia with distinct leukemogenic strengths and phenotypes.[1] *NUP98*-related fusions alone require long periods of time to induce AML, although these fusions induce MDS or MPN by impaired myeloid differentiation.[2] Cooperation between BCR-ABL (which enhances proliferation) and NUP98-fusion (which inhibits differentiation) lead to CML blast crisis.[2] Moreover, the *WT1* mutations were clustered within the DNA binding domain, and were subsequently considered to impair the ability of DNA to bind to target genes associated with apoptosis, cell cycle or cellular proliferation.[8] These results suggest that a high frequency of cell proliferation gene mutations may contribute to leukemogenesis in *NUP98*-related leukemia, and that simultaneous occurrence of *FLT3-ITD* and *WT1* aberrations may have an important role in the clinical outcome of *NUP98*-related leukemia.

Conflict of interest

The authors declare no conflict of interest.

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Serum Eosinophil Cationic Protein and 27 Cytokines/Chemokines in Acute Exacerbation of Childhood Asthma

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Key Words

Asthma · Childhood asthma · Corticosteroids · Eosinophil cationic protein · Eosinophils · IL-5

Abstract

Background: Little information is available on eosinophil activation and cytokine/chemokine responses in childhood asthma, thus we examined serum eosinophil cationic protein (ECP) and 27 types of cytokines/chemokines in acute exacerbation of asthma (acute asthma) and stable asthma. ***Methods:*** We determined peripheral eosinophil count, and the serum levels of ECP and 27 types of cytokines/chemokines (IL-1β, IL-1ra, IL-2, -4, -5, -6, -7, -8, -9, -10, -12, -13, -15 and -17, IFN-γ, IP-10, TNF-α, GM-CSF, G-CSF, MCP-1, MIP-1α and -1β, eotaxin, RANTES, PDGF-bb, FGF basic and VEGF) using a multiplex bead-based assay in 85 acute and 79 stable asthma patients, and 14 controls. We also examined the effects of systemic corticosteroids on these responses in acute asthma. ***Results:*** The serum levels of ECP, IL-5, -6, -8 and -10, G-CSF, MCP-1, IL-1ra and IP-10 were significantly elevated in acute compared with stable asthma. Similarly, serum levels of ECP, IL-5 and IP-10 were significantly higher in acute asthma than in controls. Furthermore, in the acute phase, elevated serum levels of ECP, IL-5, IL-6, IL-1ra and IP-10, but not IL-8, IL-10, G-CSF and MCP-1 were significantly reduced after treatments that included systemic corticosteroids. ***Conclusion:*** Eosinophil activation could be induced by acute exacerbation of childhood asthma.

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Introduction

Eosinophils are important effector cells in host defense against parasites and in allergic diseases such as bronchial asthma. In allergic inflammation, mediators released from epithelial or inflammatory cells induce the migration of eosinophils from the blood into the affected tissues. After migration, eosinophils are activated by appropriate stimuli resulting in the release of inflammatory mediators that include arachidonic acid metabolites, such as platelet-activating factor or cystenyl leukotrienes, oxygen radicals, cytokines/chemokines and toxic cationic granule proteins, such as major basic protein, eosinophil peroxidase, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin [1, 2]. Evidence suggests that eosinophils and their product, ECP, can serve as markers of disease activity in asthma [3]. On the other hand, several cytokines/chemokines are elevated in acute exacerbation of childhood asthma [4, 5].

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The purpose of this study was to investigate changes in the serum levels of ECP and several cytokines and chemokines in acute exacerbation of childhood asthma, and to evaluate eosinophil activation in children with bronchial asthma.

Patients and Methods

Patients

The subjects who visited and/or were hospitalized with acute respiratory symptoms (acute asthma: 54 males and 31 females; mean age, 3.9 years), or who visited for regular physical examinations and treatment (stable asthma: 48 males and 31 females; mean age, 5.0 years) at the Gunma Children's Medical Center between November 1, 2003, and October 31, 2006, were enrolled in this study. All recruited patients had a history of three or more separate episodes of recurrent wheezing and documented evidence of wheezing by auscultation. The diagnosis of asthma and its severity in patients with acute exacerbations (mild attack, 4; moderate attack, 79, and severe attack, 2) were defined according to the guidelines of the Japanese Society of Pediatric Allergy and Clinical Immunology [6]. Acute asthma was diagnosed by the emergency department physician based on the presence of wheezing and increased difficulty of breathing. Briefly, a mild attack was defined as mild wheezing, with stable disease and no dyspnea, and an $S_pO_2 \geq 96\%$; a moderate attack was defined as wheezing with dyspnea, apparent retraction and an S_pO_2 from 92 to 95%, and a severe attack was defined as more severe wheezing and dyspnea, and an $S_pO_2 \leq 91\%$. Patients with mild-moderate attacks were treated with intravenous infusion, and salbutamol and disodium cromoglycate inhalation (three times/day). Patients who experienced a severe attack were treated with isoproterenol inhalation instead of salbutamol. When S_pO_2 was $\leq 95\%$, oxygen therapy was started. Most patients with acute asthma were treated with intravenous prednisolone for 3–5 days.

The patients with acute asthma had a history of a cold prior to exacerbation. We excluded children with obvious bacterial infections, congenital heart diseases, chronic lung diseases, children who presented with a foreign body or had signs of severe infection, and those who were immunosuppressed, as these complications could interfere with the assessment of asthma-related outcome measures. Stable asthma was defined as physician-diagnosed asthma which was stable (no wheezing) at the time of examination and for at least 3 months prior to the examination. Previous treatment consisted of short-acting β_2-agonists and/or long-term treatment for asthma control without systemic corticosteroids. The control group included 14 healthy children (9 males and 5 females; mean age, 3.6 years) with no symptoms of wheezing at the time of examination. Exclusion criteria for the controls were immunosuppression, the presence of other respiratory tract symptoms or a history of previous wheezing and asthma. We attempted to match asthma patients and controls for age and sex. This study was approved by the Ethics Committee of the Gunma Children's Medical Center. Informed consent was obtained from the parents, and assent was obtained from the children when they were old enough (usually >9 years old).

Serum ECP and Cytokines/Chemokines

We determined peripheral eosinophil count and the serum levels of ECP and 27 types of cytokines/chemokines, interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, -4, -5, -6, -7, -8, -9, -10, -12, -13, -15 and -17, interferon (IFN)-γ, IFN-γ-induced protein (IP)-10, tumor necrosis factor (TNF)-α, granulocyte-macrophage (GM) colony-stimulating factor (CSF), granulocyte CSF (G-CSF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and -1β, eotaxin, RANTES (regulated on activation, normal T cell expressed and secreted), platelet-derived growth factor-bb, basic fibroblast growth factor and vascular endothelial growth factor, from 85 acute and 79 stable asthma patients who were not on systemic corticosteroids at the time of examination, and from 14 controls. To analyze the effects of treatment including systemic corticosteroids, these parameters were measured both at the time of admission and at the time when wheezing disappeared (mean days after admission = 7.3), and the levels before and after treatment were compared for each factor in 56 acute asthmatic patients. ECP content in serum was measured with a fluoroenzyme immunoassay kit (Pharmacia, Uppsala, Sweden). Serum cytokines/chemokines were determined by a multicytokine detection system (Bio-Rad, Hercules, Calif., USA), following the manufacturer's instructions, using a Luminex System (Luminex, Austin, Tex., USA) and then quantified using Bio-Plex software (Bio-Rad).

Statistical Analysis

Data were expressed as means ± SEM. Paired and unpaired data were analyzed by Wilcoxon and Mann-Whitney U test, respectively. A statistically significant difference was defined as $p < 0.05$ (two-tailed). All analyses were performed with a statistical software package (SPSS for Windows, version 12.0; SPSS Japan, Tokyo, Japan).

Results

Serum ECP and Cytokines/Chemokines

The serum levels of ECP, IL-5, -6, -8 and -10, G-CSF, IL-1ra, MCP-1 and IP-10 were significantly elevated in acute compared with stable asthma (table 1). By contrast, eosinophil counts were slightly but significantly decreased in acute asthma compared with stable asthma. Similarly, serum levels of ECP, IL-5 and IP-10 were significantly higher in acute asthma compared with controls. Furthermore, only ECP was significantly elevated in stable asthma compared with controls. No significant difference was found between mild, moderate and severe attacks in acute asthma for any parameter (data not shown). Finally, in the acute phase, elevated serum levels of ECP, IL-5, IL-6, IL-1ra and IP-10 were significantly reduced after treatment with systemic corticosteroids (fig. 1). However, treatment did not significantly affect elevated serum levels of IL-8, IL-10, G-CSF and MCP-1 in the acute phase (data not shown).

Table 1. Elevated ECP and cytokines/chemokines in patients with acute compared with stable asthma or controls

	Acute asthma (n = 85)	Stable asthma (n = 79)	Control (n = 14)
Eosinophils	250 (0–2,846)	336 (0–1,386)	204 (0–1,976)
ECP	15.8 (3.0–109.0)[a, d]	12.6 (2.6–70.9)[d]	7.3 (3.2–80.6)
IL-1β	6.27 (0.01–52.71)	7.09 (0.24–57.72)	7.72 (2.18–31.04)
IL-1ra	406.26 (45.18–4,556.18)[b]	201.07 (46.49–3,195.79)	213.08 (100.63–1,253.91)
IL-2	6.86 (0.43–248.75)	8.63 (0.60–232.71)	8.87 (3.57–12.01)
IL-4	15.01 (1.62–151.46)	16.18 (0.96–84.63)	15.54 (2.04–73.31)
IL-5	4.97 (0.07–118.44)[b, c]	2.23 (0.07–19.33)	3.10 (0.02–7.64)
IL-6	80.49 (12.49–794.13)[b]	55.44 (1.10–1,138.54)	59.86 (14.12–238.48)
IL-7	15.34 (1.46–42.8)	14.35 (0.83–29.21)	16.87 (0.83–27.14)
IL-8	12.84 (0.21–171.26)[b]	6.19 (0.36–27.54)	9.38 (0.36–38.39)
IL-9	58.75 (5.44–2,036.17)	51.87 (20.30–731.00)	39.00 (29.37–72.90)
IL-10	16.58 (0.13–145.43)[b]	3.74 (0.13–54.52)	19.82 (0.35–64.32)
IL-12	5.60 (0.54–91.06)	6.77 (0.94–256.53)	6.99 (0.29–52.25)
IL-13	3.09 (0.07–26.26)	3.28 (0.12–9.69)	3.62 (1.70–4.63)
IL-15	10.08 (1.20–71.27)	8.77 (1.23–201.70)	16.99 (3.38–90.08)
IL-17	7.35 (0.09–51.07)	8.70 (0.41–43.58)	0.06 (0.06–0.06)
IFN-γ	129.82 (3.53–529.57)	134.87 (0.263–401.78)	149.47 (44.77–1,061.34)
IP-10	1,395.52 (158.06–23,554.11)[b, d]	439.48 (140.80–1,325.22)	541.85 (330.86–1,981.86)
TNF-α	19.51 (0.07–682.83)	32.54 (0.25–97.92)	30.11 (0.25–392.13)
GM-CSF	33.82 (0.20–962.04)	21.80 (2.33–327.07)	22.23 (0.56–71.99)
G-CSF	43.88 (2.24–1,341.93)[a]	34.71 (0.24–275.39)	38.03 (19.42–129.62)
MCP-1	54.96 (13.01–254.60)[a]	46.21 (5.50–102.64)	62.98 (30.23–96.89)
MIP-1α	12.26 (1.47–46.77)	12.61 (4.72–25.20)	12.95 (10.60–14.79)
MIP-1β	80.72 (6.00–275.91)	99.40 (18.37–304.12)	116.91 (15.26–168.02)
Eotaxin	80.95 (35.48–440.78)	97.61 (18.30–949.34)	112.81(69.83–577.43)
RANTES	5,339.63 (2,146.85–114,340.39)	9,089.88 (4,994.69–25,210.45)	5,831.70 (4,632.50–7,030.89)
PDGF-bb	7,273.09 (433.33–19,544.26)	7,730.38 (3,657.11–15,311.06)	6,540.45 (2,622.59–12,493.89)
FGF-basic	68.63 (15.39–471.64)	96.76 (24.72–280.66)	71.97 (35.20–156.77)
VEGF	115.74 (5.22–729.05)	107.33 (20.14–1,287.23)	65.81 (18.13–270.06)

Medians (ranges), Mann-Whitney U test, [a] $p < 0.05$; [b] $p < 0.001$, vs. stable asthma; [c] $p < 0.05$; [d] $p < 0.01$, vs. control. PDGF-bb = Platelet-derived growth factor-bb; FGF = fibroblast growth factor; VEGF = vascular endothelial growth factor. Eosinophils: n/mm^3; ECP: ng/ml, and cytokines/chemokines: pg/ml.

Discussion

Although previous reports showed serum ECP [7–10] and serum or plasma cytokine/chemokine production [7, 10–19] in acute exacerbations of asthma, this is the first report to show the profiles of ECP and 27 cytokines/chemokines in serum samples of patients with acute and stable asthma, and in controls. In this study, serum ECP was significantly elevated in acute asthma compared with stable asthma and controls. Similarly, in previous reports, serum levels of ECP were significantly higher in children with acute asthma than in those with stable asthma and controls [7, 8]. These results suggest that acute exacerbation of asthma might enhance eosinophil activity, e.g. degranulation.

Oymar et al. [7] also reported that the serum level of IL-5 was significantly elevated in children with acute asthma. In an analysis of the correlation between each factor using data from patients with acute asthma, a significant association was found between peripheral blood eosinophil count and serum ECP ($r = 0.541$, $p < 0.0001$), and between ECP and IL-5 ($r = 0.310$, $p = 0.0015$). These results suggest that eosinophil activation during acute asthma is mediated at least in part by IL-5.

Th2 cytokines are thought to mediate most of the allergic inflammatory responses associated with atopic asthma. However, the Th1-related chemokine IP-10/CXCL10 was the predominant chemokine measured during human allergic pulmonary late-phase reactions [20]. In our study, the serum level of IP-10 was also signifi-

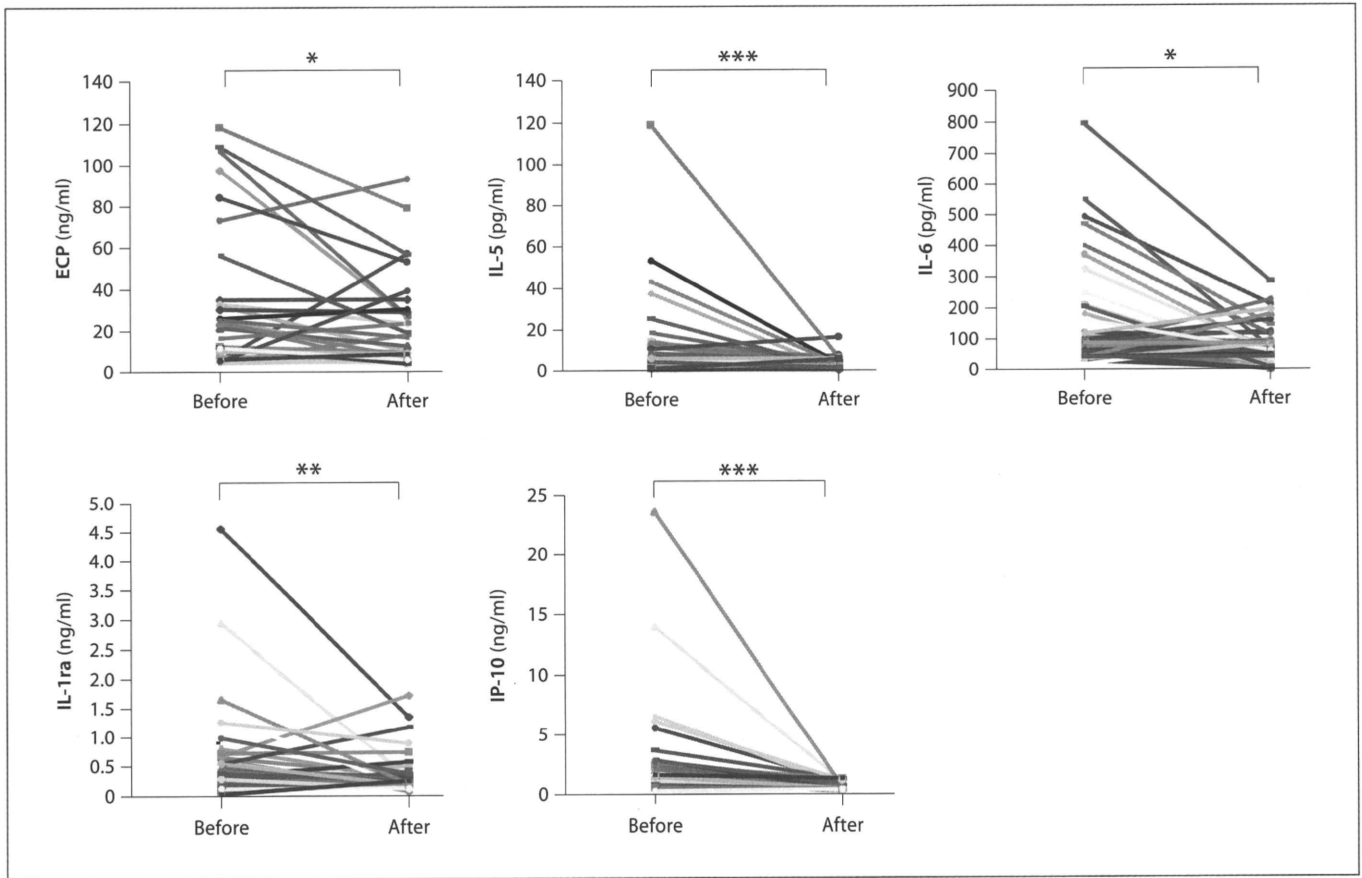

Fig. 1. Effect of treatment that included systemic corticosteroids on elevated serum levels of ECP and cytokines/chemokines in acute asthma. In the acute phase, elevated serum levels of ECP, IL-5, IL-6, IL-1ra and IP-10 were significantly decreased after treatment. Differently colored lines show the values for each subject. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, before vs. after treatment; Wilcoxon test.

cantly higher in acute asthma than in stable asthma and controls. In similar reports, serum or plasma levels of IP-10 were increased in acute asthma, specifically in cases of rhinovirus infection [14, 15], suggesting that IP-10 is a novel marker of acute exacerbation of asthma induced by rhinoviruses. However, these studies failed to clarify the viral pathogen. IL-1ra is a potent anti-inflammatory cytokine [21]. Previously, serum levels of soluble TNF-receptor I and II, and IL-1ra obtained during bronchial asthma attacks were higher than those from patients in stable condition [16]. This report and our results indicate that higher serum levels of IL-1ra may reflect the up-regulation of IL-1ra production in acute asthma, and that IL-1ra may contribute to TNF-α- and IL-1-mediated production of IL-1ra per se in acute asthma.

IL-8 and G-CSF are major cytokines in the recruitment of neutrophils to the inflammatory area [22]. Tang and Chen [12] reported that the serum level of IL-8 was significantly higher in children with acute asthma and in stable asthmatics than in control subjects. In contrast to our results, the difference between acute and stable asthmatics was not significant, indicating that serum IL-8 is a poor indicator of disease activity in asthma.

Corticosteroid therapy is one of the most effective treatments for asthma. However, evidence suggests that treatment with inhaled corticosteroids does not improve airway inflammation induced by rhinovirus infection [23]. On the other hand, oral glucocorticoids improve lung function and decrease serum IL-6, soluble ICAM-1 and ECP levels [10]. In this study, treatment comprising systemic corticosteroids resulted in decreases in serum levels of ECP, IL-5, IL-6, IL-1ra and IP-10, but not IL-8, IL-10, G-CSF and MCP-1. In previous studies, systemic corticosteroid treatment of children with asthma signifi-

cantly reduced serum concentrations of ECP [24–26], IL-5 [26] and IP-10 [12]. Furthermore, systemic corticosteroids significantly reduced both IL-5 gene expression and serum levels of ECP in acute compared with stable asthma [27]. These results suggest that systemic corticosteroids might reduce eosinophil activation via IL-5 production. However, since this investigation was not a case-control study of systemic corticosteroids, a study designed to discover the exact effects of systemic corticosteroids is needed.

Further investigation into the mechanisms behind the association between asthma exacerbations and eosinophil activation is required.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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TABLE I. Sensitivity and specificity* for Ara h 2 and whole peanut extract

Test	Cutoff point (kU_A/L)	Sensitivity (%)	Specificity (%)	Correctly classified (%)
Ara h 2	0.30	100.00	90.20	93.75
	0.32	100.00	94.12	95.00
	0.35	100.00	96.08	97.50
	0.38	96.55	96.08	96.25
	0.40	93.10	98.04	96.25
	0.55	93.10	100.00	97.50
	0.87	89.66	100.00	96.25
Whole extract	0.35	96.55	26.92	51.85
	3.91	79.31	84.62	82.72
	5.00	75.86	90.38	85.19
	5.30	75.86	94.23	87.65
	5.96	72.41	94.23	86.42
	7.81	72.41	96.15	87.65
	15.00	55.17	96.15	81.48
	43.86	34.85	98.08	75.31

Analysis included all children with available data (81 for sIgE to whole peanut extract and 80 for sIgE to Ara h 2).
*Sensitivity refers to the proportion of subjects who have peanut allergy and give positive test results. Specificity refers to the proportion of subjects without the target condition and a negative test result for peanut allergy.

peanut allergy and 50 are peanut-tolerant. By using sIgE to component Ara h 2 with a cutoff point of 0.35 kU_A/L, all children with peanut allergy would be correctly classified. The specificity of this test is given as 96.1% (Table I). In this example we expect 2 children who are not allergic to peanuts to be misclassified as having peanut allergy and the other 48 children to have a negative result. By using this cutoff point, 97.5% of the population is correctly classified. A similar proportion of children would be correctly classified by using a cutoff point of 0.55 kU_A/L; however, in this case 3 children with peanut allergy would be misclassified as tolerant. This cutoff point corresponds to a gain in specificity (100%) but a loss in sensitivity (93.1%). Given the importance of not misdiagnosing children with peanut allergy as being tolerant, we propose that the optimal cutoff point in our population is 0.35 kU_A/L.

The cutoff for whole peanut sIgE of 5.30 kU_A/L provides the maximum proportion of correctly classified subjects (87.6%), with a sensitivity of 75.9% and a specificity of 94.2%. However, approximately 24% of children with peanut allergy would be inappropriately classified as peanut-tolerant. The cutoff of 15 kU_A/L has excellent specificity, with 96.2% of children at greater than this level being correctly classified as allergic; however, this decision point has relatively poor sensitivity, with almost half of the subjects with peanut allergy being classified as tolerant. These data suggest that in our population the quantification of whole peanut sIgE has lower accuracy in discriminating peanut allergy from tolerance compared with quantification of sIgE to Ara h 2.

In conclusion, having identified sIgE to Ara h 2 as an important predictor of clinical reactivity to peanut using microarray technology,[5] we have now demonstrated the value of its quantification using a routinely available laboratory test. Among school-aged children in the United Kingdom, a cutoff of 0.35 kU_A/L Ara h 2 IgE confers 100% sensitivity and 96.1% specificity. By using this cutoff point, 97.5% of the subjects in our study population were correctly classified, with all children with peanut allergy given the correct classification. The importance of Ara h 2 has been suggested in studies from other Central and Northern European countries[7,8]; however, in other populations and geographic areas, IgE to other components might be relevant (eg, Ara h 9 in the Mediterranean[9]). Our findings need to be replicated in other populations and age groups before general application.

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Four distinct subtypes of non–IgE-mediated gastrointestinal food allergies in neonates and infants, distinguished by their initial symptoms

To the Editor:

Although most food allergies are IgE-mediated, there are a number of non–IgE-mediated gastrointestinal food allergies that affect mainly infants and young children.[1,2] Because most such

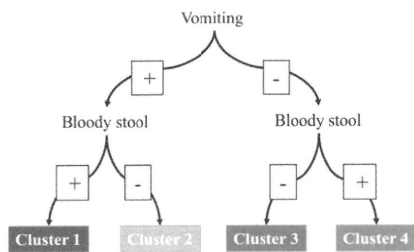

FIG 1. Tree analysis using 2 variables (vomiting and bloody stool at initial presentation) enables assignment of patients into 4 clusters.

patients develop the allergy more than 2 hours after ingestion of the offending food and show negative skin prick tests and/or absence of serum specific IgE against the offending food, these allergies are thought to be cell-mediated. However, the precise pathogenetic mechanisms of these disorders remain poorly understood. Several investigators have described different subtypes of non–IgE-mediated gastrointestinal food allergies: food protein-induced enterocolitis syndrome (FPIES),[3] food protein-induced proctocolitis syndrome (hereafter referred to as "proctocolitis"),[4] food protein-induced enteropathy syndrome (hereafter referred to as "enteropathy"),[5] celiac disease, and allergic eosinophilic gastroenteropathies.

Presumably because the main target organ of these syndromes is the gastrointestinal tract, patients with non–IgE-mediated gastrointestinal food allergies often exhibit similar symptoms, such as vomiting and diarrhea. However, it remains unclear whether these syndromes have the same pathogenesis and merely differ in severity, or whether the pathogenesis of each is distinct, meaning that they should be classified as separate clinical entities.

We applied cluster analysis to the clinical and laboratory findings to characterize these non–IgE-mediated food allergies and determine whether they are made up of distinct clinical entities. A total of 176 patients with detailed clinical records who had been registered in the database of the Japanese Research Group for Neonatal, Infantile Allergic Disorders from 2007 to 2010 were enrolled. Among them, 136 patients fulfilled 3 of the Powell[6] criteria: (1) a switch to therapeutic milk led to resolution of symptoms, (2) differential diagnosis from other disorders was possible, and (3) there was verified body weight gain. Definitive diagnosis was possible for 46 patients by oral food challenge tests that were performed after complete resolution of the initial symptoms (see this article's Fig E1 in the Online Repository at www.jacionline.org). These 46 patients were subjected to further analysis. Details of food challenge test are available in this article's Food challenge test, method section in the Online Repository at www.jacionline.org. Our total cohort included 15 patients who developed the most severe reactions, including ileus, shock, and developmental retardation. The clinical characteristics of those patients are summarized in this article's Table E1 in the Online Repository at www.jacionline.org. Because of the medical and ethical justification, even though these patients fulfilled 3 elements of the Powell[6] criteria, oral challenge tests were not performed. Thus, these patients were excluded from this cluster analysis of 46 patients. This study was approved by the Ethics Committee of the National Center for Child Health and Development.

We omitted clinical and laboratory findings found only in a few patients and finally selected 5 variables: birth weight, age at first presentation (days after birth), severity of vomiting (ranked as 0, none; 1, 1-2 times a day; 2, 3-5 times a day; and 3, more than 5 times a day or bilious vomiting) and severity of bloody stool (0, none; 1, spotty; 2, intermediate; and 3, massive) at first presentation, and milk-specific IgE antibody titer (class 0-6). Unsupervised cluster analysis and discriminant analysis were performed by using SPSS version 18 software (SPSS, Inc, Chicago, Ill). The Wald minimum-variance hierarchic clustering method was performed by using an agglomerative (bottom-up) approach and Ward's linkage. The squared Euclidean distance was used as a proximity measure. Values were transformed by a maximum magnitude of 1. ANOVA, the Tukey-Kramer test, and the χ^2 test were used for parametric continuous, nonparametric continuous, and categoric variables. As a result, the 46 definitively diagnosed patients were classified into 4 distinct clusters, and a dendrogram was generated (see this article's Fig E2 in the Online Repository at www.jacionline.org).

Stepwise discriminate analysis identified the 2 strongest discriminatory variables for cluster assignment: vomiting and bloody stool (Fig 1). Cluster 1 was the patient group with vomiting and bloody stool at initial presentation. Cluster 2 had vomiting but not bloody stool. Cluster 3 had neither vomiting nor bloody stool. Cluster 4 had bloody stool but not vomiting. One patient initially assigned to cluster 3 in fact had clear bloody stool, and was thus reassigned to cluster 4 in accordance with Fig 1. As a result, clusters 1 through 4 consisted of 14, 16, 5, and 11 patients, respectively.

Table I presents the demographic data for each cluster. Cluster 3 showed a significantly lower birth weight and later onset of disease. Clusters 1 and 4 both had bloody stool, but they had normal birth weight and a somewhat earlier onset (median of 7 days after birth).

The laboratory data generated within the initial several days after onset showed that the peripheral blood eosinophil ratio was high in all clusters, with no significant differences among them. In contrast, eosinophils were found in the stool mainly of patients in clusters 1 and 4, in which all patients, by definition (Fig 1), had bloody stool. The presence of eosinophilia suggests that patients with non–IgE-mediated gastrointestinal food allergies tend to have a T_H2-prone immune deviation at baseline, but some additional factors such as overproduction of eosinophil-attracting chemokines are probably necessary to induce immune responses involving eosinophils in the gut (see this article's Fig E3 in the Online Repository at www.jacionline.org).

A positive milk-specific IgE antibody titer was observed in 37% of the patients, with no statistically significant differences among any of the clusters. In addition, almost all symptoms at initial presentation as well as in oral food challenge tests began to manifest at more than 2 hours after ingestion of the offending food, whereas no patients developed typical IgE-mediated symptoms such as urticaria or wheeze. These results strongly suggest that the presence of milk-specific IgE antibody neither causes the gastrointestinal symptoms nor rules out a diagnosis of non–IgE-mediated gastrointestinal food allergy.

One of the most notable findings of this study was the remarkably high reproducibility of symptoms provoked in the oral food challenge tests and those found at the initial presentation in all 4 clusters, even though the oral challenge tests were performed several months after the initial presentation (Table I). This observation suggests that the upper or lower gastrointestinal tract–specific hypersensitivity and perhaps the responsible

TABLE I. Demographic data of the patients (total = 46) whose diagnosis was confirmed by oral food challenge tests

Clinical characteristics		Cluster 1 (n = 14)		Cluster 2 (n = 16)		Cluster 3 (n= 5)		Cluster 4 (n = 11)	P value
Birth weight (g)		2642 (2410-3030)		2745 (2223-3079)		1008 (907-2491)		2678 (2512-3170)	.03*
Male/female (n)		6/8		9/7		2/3		5/6	.95
Initial presentation									
Day of onset		7.5 (3-23)		16.5 (9.5-33.5)		37 (8.5-132)		7 (2-56)	.17
Vomiting (%)		100		100		0		0	.000*
Bloody stool (%)		100		0		0		100	.000*
Fever (%)		7.1		18.8		20.0		0	.45
(Laboratory data)†	n		n		n		n		
Blood eosinophil ratio (%)‡	13	15 (3.0-23)	14	7 (3.9-19.3)	5	27 (3.2-39.3)	11	14 (4.5-25)	.63
WBC ($\times 10^3$/mL)§	13	18.4 (13.7-22.7)	14	15.7 (11.4-21.9)	5	21.8 (11.0-27.7)	11	13.1 (8.2-18.3)	.64
Total IgE (IU/mL)‖	14	5.2 (4.8-28.3)	16	11.4 (5.0-80.8)	5	7.4 (5.5-653.5)	10	5.0 (2.0-5.8)	.36
Positive for milk-specific IgE (class ≥1) (%)	14	57	16	37.5	5	40	11	9	.28
C-reactive protein (% positive, ≥0.5)	13	46	14	50	5	40	10	30	.47
Stool eosinophil (% positive)	8	50	6	33	3	0	7	100	.01*
Diet (reaction to each milk, %)									
Cow's milk	14	100	16	100	5	100	10	100	1.00
Breast milk	8	38	7	0	2	50	7	27	.40
Hydrolyzed formula	9	0	10	20	2	0	8	63	.02*
Oral food challenge test									
Onset of reaction (h)		6 (1.8-12)		10 (2-24)		48 (24-60)		24 (24-48)	.17
Vomiting (%)		85.7		81.3		0		9.1	.000*
Bloody stool (%)		28.6		6.3		0		72.7	.001*
Diarrhea (%)		21.4		31.3		60.0		18.2	.33

WBC, White blood cell count.
Data are shown as the median and the interquartile range.
$^*P < .05$.
†*n,* Number with medical records.
‡Normal range of blood eosinophils is 0% to 4%. However, it is known to rise to some degree in the neonatal period, especially in low-birth-weight infants.[10]
§Normal range of WBC in neonatal period is 7.0 to 25.0 $\times$ $10^3/\mu L$.
‖Normal range of total IgE in infantile period is less than 20 IU/mL.

immune cells remain in the same part of the gastrointestinal tract even after several months' remission.

Because the patients in clusters 1 and 2 had vomiting that was provoked at relatively early time points, they are likely to be diagnosed as having FPIES, although the bloody stool and eosinophilia seen mainly in cluster 1 patients were not emphasized in earlier reports.[7,8] The nearly simultaneous manifestation of vomiting and bloody stool suggests that FPIES may affect both the upper and lower gastrointestinal tracts.

The main symptoms of the patients in cluster 3 were poor weight gain and diarrhea and were similar to those found in patients with enteropathy. The significantly lower birth weight and marked eosinophilia characteristically found in cluster 3 patients imply the involvement of immature gastrointestinal function in the pathogenesis of this syndrome.

Bloody stool was the main symptom of the patients in cluster 4. Some patients in this cluster had no systemic manifestation other than bloody stool, whereas others also had diarrhea and/or poor weight gain. Therefore, these patients may be diagnosed as having proctocolitis or early onset of allergic eosinophilic gastroenteropathies, respectively. However, the pathogenetic similarity and/or disparity of proctocolitis and allergic eosinophilic gastroenteropathies need to be studied further.

In our cohort, 3 children with exclusive breast-feeding have developed FPIES. This information is available in this article's Breast-feeding and FPIED section in the Online Repository at www.jacionline.org.

Elevated serum C-reactive protein levels were found in 30% to 50% of patients with non–IgE-mediated gastrointestinal food allergies. In addition, some patients developed a fever during oral food challenge tests, suggesting that TNF-α and other proinflammatory cytokines may be involved in the pathogenesis of these syndromes.[9]

To confirm the results of cluster analysis, we performed the same analysis for the aforementioned 136 patients who fulfilled 3 of the Powell[6] criteria (consisting of the 46 patients definitively diagnosed by oral food challenge and 90 patients not subjected to oral food challenge; Fig E1). We obtained exactly the same results: the patients were assigned to 4 clusters in accordance with the tree analysis shown in Fig 1. The patients' demographics (see this article's Table E2 in the Online Repository at www.jacionline.org), birth weight (see this article's Fig E4 in the Online Repository at www.jacionline.org) and peripheral blood eosinophils (see this article's Fig E5 in the Online Repository at www.jacionline.org) confirmed the earlier cluster analysis findings.

In our ongoing cohort, 52% of the patients acquired tolerance to the offending food by 1 year of age, 88% by 2 years, and 94% by 3 years. Therefore, assuming that identification and elimination of the offending food had been done properly, it can be assumed that most patients outgrew their allergy by the age of 2 to 3 years. On the other hand, just like patients with severe IgE-mediated food allergy, patients with non–IgE-mediated gastrointestinal food allergies may develop severe reactions

(Table E1). Thus, early diagnosis is very important, and refinement of the diagnostic method is truly necessary.

Our findings clearly demonstrated that patients with these non–IgE-mediated gastrointestinal food allergies showed similar T_h2-prone laboratory data (eosinophilia and presence of specific IgE antibody), but the disease entities of each cluster had distinct clinical features and may have different pathogenetic mechanisms.

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FOOD CHALLENGE TEST, METHOD

Generally, oral challenge tests were performed at 4 to 6 months of age. First, 4 mL milk/kg body weight was administered. If no reaction occurred, the dose of milk was increased daily until symptoms manifested. If the reaction had been evoked by a very small volume of milk in the initial presentation, the test was started using a lesser volume to avoid a serious reaction. Because of the medical and ethical justification for oral food challenge tests, patients with the most severe reactions were excluded from the initial cluster analysis. Their clinical characteristics are summarized in Table E1.

BREAST-FEEDING AND FPIES

Six of the 46 patients were exclusively breast-fed. Three of them were included in cluster 1 and can be diagnosed as FPIES. Those 3 patients showed a positive reaction to cow's milk as well as breast milk even after their mothers stopped consuming milk products. These patients also developed symptoms when orally challenged with rice and/or soy. Therefore, these findings indicate that not only proctocolitis but also FPIES can develop even in children who are exclusively breast-fed. A recent case report supports our findings.[E1]

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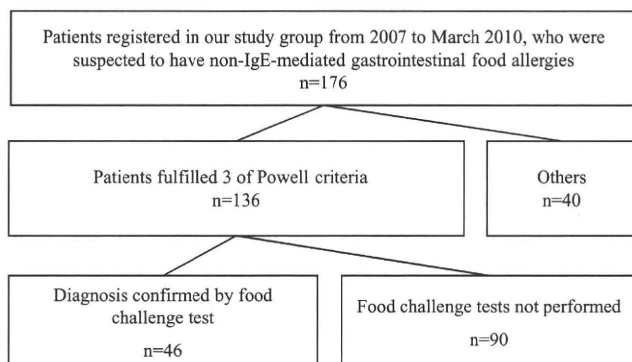

FIG E1. A total of 176 patients with gastrointestinal symptoms who were suspected of having non–IgE-mediated allergy from 1999 to 2009 were registered by doctors of the Japanese Research Group for Neonatal, Infantile Allergic Disorders. Of them, 136 patients fulfilled elements 1 through 3 of the Powell criteria. Forty-six patients underwent food challenge tests and had a positive result, whereas the remaining 90 patients were not tested. Seventeen patients showed no reaction in the oral challenge tests. However, it was unclear whether this was because the patients had outgrown their allergy or because of misdiagnosis. Those 17 patients were excluded from further analysis in this study.

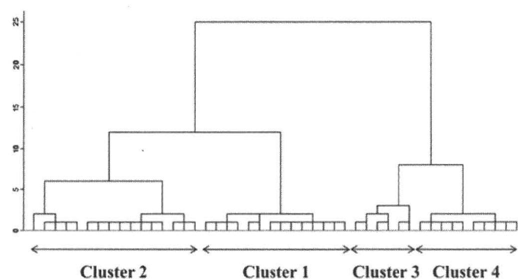

FIG E2. The 46 patients definitively diagnosed with non–IgE-mediated food allergies were analyzed for 5 variables by using an agglomerative (bottom-up) approach and Ward's linkage, and a dendrogram was generated.

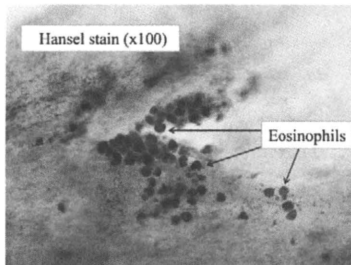

FIG E3. Detection of accumulations of eosinophils in the stool mucus. The mucous part of the stool was thinly smeared on a glass slide and stained by using Hansel stain. The stool sample was taken from a patient in cluster 2 after a positive food challenge test. Representative images were found in a total of 13 patients (Table I).

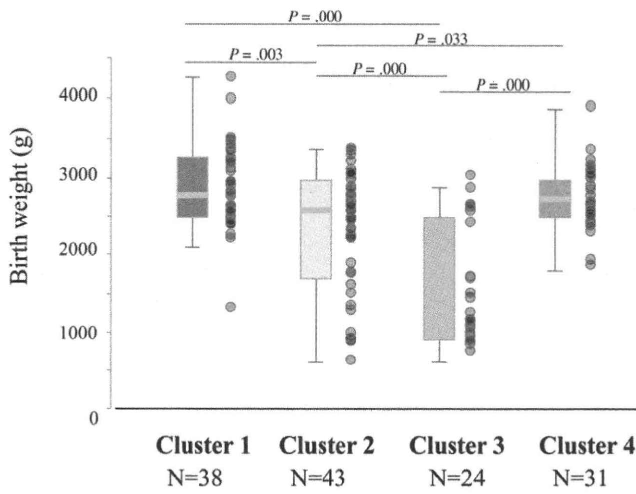

FIG E4. The birth weights in each cluster of the 136 patients who fulfilled 3 elements of the Powell criteria for a non–IgE-mediated allergy are shown.[E2] The birth weights in cluster 3 were significantly lower than in the other clusters. Moreover, 2 subgroups seem to be identified in cluster 3: a lower birth weight group and a normal birth weight group.