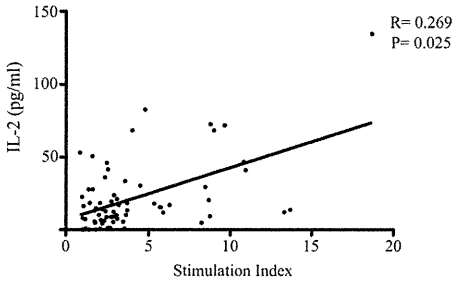

FIG E2. IL-2 concentrations in culture supernatant of cow's milk protein–stimulated PBMCs correlated significantly with antigen-specific lymphoproliferation. PBMCs from children with gastrointestinal food allergies were stimulated separately with 100 μg/mL of each of 5 LPS-depleted milk protein preparations in the absence of serum for the antigen-specific lymphoproliferation assay and in the presence of 5% autologous plasma for the IL-2 production assay. The stimulation index was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm), and the highest stimulation index shown among the 5 tested protein preparations was used as that patient's data in the plot. Even under slightly different culture conditions, antigen-specific lymphoproliferation and antigen-specific IL-2 production were significantly correlated ($r = 0.269$, $P = .025$).

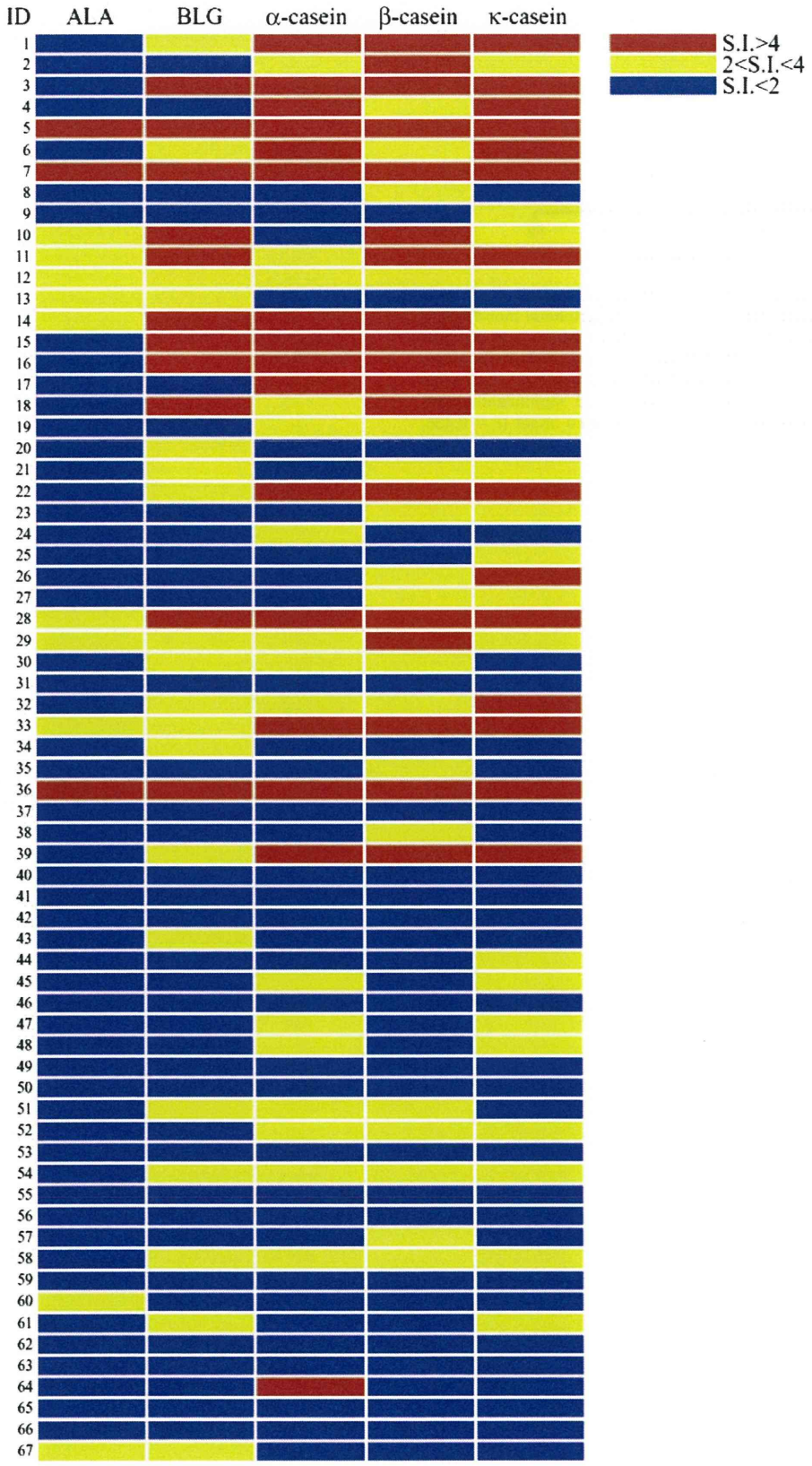

FIG E3. The milk protein component causing the most prominent tritiated thymidine uptake varied among the patients. PBMCs from children with gastrointestinal food allergies were stimulated separately with 100 μg/mL of each of 5 LPS-depleted milk protein preparations in the absence of serum. Lymphoproliferation was measured based on tritiated thymidine uptake. The stimulation index *(S.I.)* was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm). For

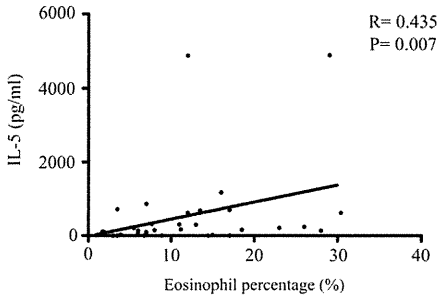

FIG E4. IL-5 concentration in the culture supernatant of cow's milk protein–stimulated PBMCs correlated significantly with the peripheral blood eosinophil percentage. PBMCs from children with gastrointestinal food allergies were stimulated separately with 100 μg/mL of each of 5 LPS-depleted cow's milk protein preparations in the presence of 5% autologous plasma for 6 days. Antigen-specific IL-5 production correlated significantly with the peripheral blood eosinophil percentage at disease onset (r = 0.435, P = .007).

each patient, the SI is shown for the PBMCs' response to each of the 5 milk protein preparations. Each *row* represents a single patient, and each *column* represents one of the 5 milk proteins. *ALA*, α-Lactalbumin; *BLG*, β-lactoglobulin; *blue*, SI < 2; *yellow*, 2.0 < SI < 4.0; *red*, SI > 4.

TABLE E1. Concentrations of LPS in commercially available milk protein preparations before and after treatment with a prepacked endotoxin affinity column

Cow's milk protein preparation	Before treatment (pg/mg)	After treatment (pg/mg)
α-Lactalbumin (Sigma L-6010)	184,200	14
β-Lactoglobulin (Sigma L-3908)	206,700	1,880
α-Casein (Sigma C-6780)	540	23
β-Casein (Sigma C-6905)	500	34
κ-Casein (Sigma C-0406)	400	41
LPS-depleted β-lactoglobulin (Bean Stalk Snow)	29	—

The indicated milk protein preparations were treated with a prepacked endotoxin affinity column (Detoxi-Gel; Pierce Chemical, Rockford, Ill) in accordance with the manufacturer's instructions. LPS concentrations were measured by using the limulus amebocyte lysate assay.

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REVIEW ARTICLE

JSA Best Presentation Award 2011

Gastrointestinal Food Allergy in Infants

Hideaki Morita[1,2], Ichiro Nomura[1], Akio Matsuda[1], Hirohisa Saito[1] and Kenji Matsumoto[1]

ABSTRACT

Food allergies are classified into three types, "IgE-mediated," "combined IgE- and cell-mediated" and "cell-mediated/non-IgE-mediated," depending on the involvement of IgE in their pathogenesis. Patients who develop predominantly cutaneous and/or respiratory symptoms belong to the IgE-mediated food allergy type. On the other hand, patients with gastrointestinal food allergy (GI allergy) usually develop gastrointestinal symptoms several hours after ingestion of offending foods; they belong to the cell-mediated/non-IgE-mediated or combined IgE- and cell-mediated food allergy types. GI allergies are also classified into a number of different clinical entities: food protein-induced enterocolitis syndrome (FPIES), food protein-induced proctocolitis (FPIP), food protein-induced enteropathy (Enteropathy) and eosinophilic gastrointestinal disorders (EGID). In the case of IgE-mediated food allergy, the diagnostic approaches and pathogenic mechanisms are well characterized. In contrast, the diagnostic approaches and pathogenic mechanisms of GI allergy remain mostly unclear.

In this review, we summarized each type of GI allergy in regard to its historical background and updated clinical features, offending foods, etiology, diagnosis, examinations, treatment and pathogenesis. There are still many problems, especially in regard to the diagnostic approaches for GI allergy, that are closely associated with the definition of each disease. In addition, there are a number of unresolved issues regarding the pathogenic mechanisms of GI allergy that need further study and elucidation. Therefore, we discussed some of the diagnostic and research issues for GI allergy that need further investigation.

KEY WORDS

eosinophil-associated gastrointestinal disorders (EGID), food protein-induced enterocolitis syndrome (FPIES), food protein-induced enteropathy (Enteropathy), food protein-induced proctocolitis (FPIP), non-IgE-mediated food allergy

INTRODUCTION

Food allergies continue to increase, especially in westernized countries,[1] and are now recognized as a worldwide problem. Food allergies are classified into three types: "IgE-mediated," "combined IgE- and cell-mediated" and "cell-mediated/non-IgE-mediated," depending on the involvement of IgE in their pathogenesis.[1-3]

Most food allergies that exhibit cutaneous and/or respiratory symptoms within 1 hour after ingestion of offending foods belong to IgE-mediated food allergies. The mechanisms and pathogenesis of IgE-mediated food allergies are well characterized. In brief, food-specific IgE antibodies are generated after initial exposure to food antigens and then bind to the surface of mast cells and basophils. Upon re-exposure to the offending foods, the food antigens bind to and cross-link the food-specific IgE antibodies bound to the mast cells and basophils, causing their activation and degranulation. Released mediators such as histamine and leukotrienes cause cutaneous and/or respiratory symptoms (Fig. 1).[4] These mechanisms were supported by the existence of serum IgE antibodies specific for offending foods and elevation of the serum histamine level after ingestion of offending

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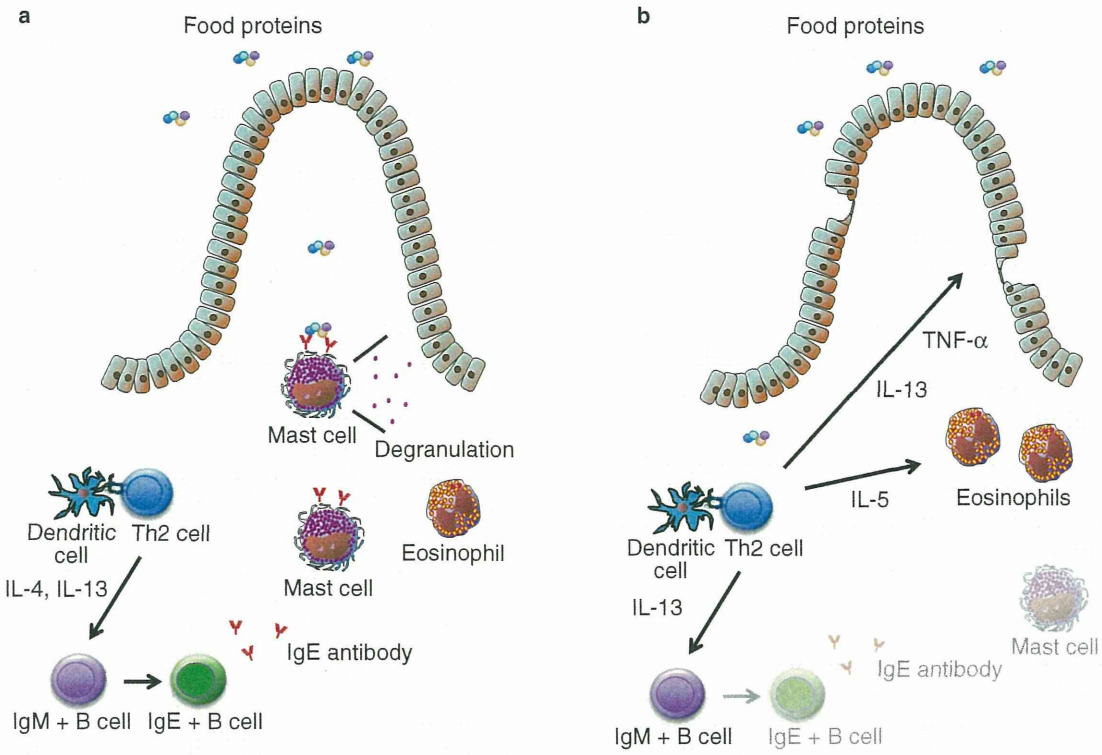

Fig. 1 Pathogenic mechanisms of IgE-mediated food allergy and gastrointestinal food allergy. (**a**) IgE-mediated food allergy. Th2 cytokines such as IL-4 and IL-13, which are produced by T cells in response to specific food antigens, induce B cells to produce food-specific IgE antibodies. The food-specific IgE antibodies bind to the surface of mast cells. Upon re-exposure to the offending food, the food-specific IgE antibodies become cross-linked on the surface of the mast cells, triggering activation and degranulation of the cells. Released mediators, such as histamine and leukotrienes, cause cutaneous and/or respiratory symptoms. (**b**) Gastrointestinal food allergy. Unlike IgE-mediated food allergy, large amounts of inflammatory cytokines such as TNF-α are also produced antigen-specifically by T cells in GI allergies. TNF-α increases intestinal permeability, which facilitates the uptake of undigested food antigens. On the other hand, as in the case of IgE-mediated food allergy, Th2 cytokines such as IL-4, IL-5 and IL-13 are produced by T cells in response to specific food antigens. However, B cells do not produce food antigen-specific IgE antibodies in most patients with GI allergy. IL-13 induces intestinal epithelial damage through activation of the tumor necrosis factor-like weak inducer of apoptosis-fibroblast growth factor-inducible molecule 14 (TWEAK-Fn14) axis. IL-5 accumulates and activates eosinophils in gastrointestinal tissues.

foods.[5] Positive skin-prick tests and significant histamine release from peripheral blood basophils after stimulation with offending food proteins *in vitro* also support these concepts. In some patients, gastrointestinal symptoms such as vomiting and diarrhea are accompanied by cutaneous and/or respiratory symptoms. However, the gastrointestinal symptoms usually appear within 1 hour after ingestion of offending foods,[1] and they are also surmised to be induced by IgE-mediated immune responses.

Unlike IgE-mediated food allergy, some patients exhibit gastrointestinal symptoms such as vomiting, diarrhea and bloody stool several hours (at least 1 hour) after ingestion of offending foods, with only rare cutaneous or respiratory manifestations. Those patients are diagnosed as having a subtype of food allergy, called gastrointestinal food allergy (GI allergy).[6] Specific IgE antibodies to offending foods are rarely detected in sera from patients with GI allergy, especially infants. Therefore, GI allergy is thought to be "cell-mediated/non-IgE-mediated" or "combined IgE- and cell-mediated" disease. However, the precise mechanisms and pathogenesis of GI allergy remain unclear.

GI allergy has classically encompassed a number of different clinical entities: food protein-induced enterocolitis syndrome (FPIES), food protein-induced proctocolitis (FPIP), food protein-induced enteropathy and eosinophilic gastrointestinal disorders (EGID).[2] In the first three, most patients are infants and rarely have detectable food-specific IgE antibodies. Therefore, they have been classified as non-IgE-

mediated diseases. On the other hand, most patients with EGID are adults and young children, and often have detectable food-specific IgE antibodies. Therefore, EGID has been classified as combined IgE- and cell-mediated disease. However, these classifications evolved from different perspectives: the clinical manifestation for the first three entities, and the histological findings for the last. Therefore, there must be some overlap between these clinical entities.[7]

In this review, we trace the history of GI allergy in the literature and summarize the defining features of each disease. We have also tried to elucidate unresolved issues regarding each disease that require further investigation.

FOOD PROTEIN-INDUCED ENTEROCOLITIS SYNDROME (FPIES) (DIETARY PROTEIN-INDUCED ENTEROCOLITIS SYNDROME)

HISTORY

In 1967, Gryboski reported a series of 21 children who developed vomiting and/or diarrhea after ingestion of milk.[8] In 1986, Powell characterized the clinical features of such patients, established diagnostic criteria and coined the term "food protein-induced enterocolitis of infancy".[9] Subsequently, Sicherer *et al.* established the term "Food Protein-Induced Enterocolitis Syndrome (FPIES)".[10]

CLINICAL FEATURES

Patients with FPIES experience repetitive vomiting, starting one or two hours after ingestion of offending foods, followed by diarrhea.[9-16] However, these patients do not develop acute cutaneous or respiratory symptoms, which commonly accompany IgE-mediated food allergy.[9-13,15] Although most patients develop symptoms at least one hour after ingestion of offending foods, total nine patients who have developed symptoms within one hour after ingestion of offending foods were reported.[10,17]

FPIES is sometimes accompanied by systemic symptoms such as hypotension,[9-12,14,17,18] lethargy,[9,11-13,17] pallor,[13] hypothermia,[13] bloody stool,[12,17-19] ileus,[19,20] methemoglobinemia[10,13,14,21] and thrombocytosis.[13,22] Some FPIES patients also develop a high fever, with neutrophilia. Therefore, these patients are sometimes initially mistakenly diagnosed as sepsis or surgical abdominal emergency, etc.[13,22-24]

Although comparatively acute onset after ingestion of offending foods is a characteristic feature of FPIES compared with other GI allergy entities, as stated above, some patients with FPIES exhibit a chronic clinical course.[13,25] The reason for this remains unclear.

OFFENDING FOODS

The most common causal foods of FPIES are cow's milk and soy-based formulas. However, solid foods such as rice,[13,16,17,26-29] oats,[13,17] eggs,[16,30,31] barley,[17] sweet potato,[13,17] chicken,[10,13,17,29,32,33] turkey,[10,17,32] peas,[10,17,32] bananas,[13] fish,[13,16,17,29] lamb,[13] corn,[16,34] and orange juice[35] have also been reported as causal foods. Breast feeding was previously thought to be a protective factor, but recent reports documented five patients with FPIES who reacted to cow's milk or soy protein passed through the breast milk.[25,36,37]

ETIOLOGY

A prospective population-based study in Israel reported that the incidence of milk-induced FPIES was 0.34%.[38] Age at onset of typical FPIES caused by cow's milk or soy-based formula was reported to be less than 9 months of age. However, some patients develop FPIES later than 9 months.[10-12] In addition, the mean age at onset of FPIES caused by solid foods tends to be higher than that of FPIES caused by cow's milk or soy-based formula. A recent report indicates that even adults may develop FPIES caused by solid foods.[39]

DIAGNOSIS

Powell initially defined FPIES based on four criteria.[9] (1) Disappearance of the symptoms of vomiting and diarrhea, and of blood and leukocytes in the stool, after all antigens are removed from the diet. (2) No other cause for the colitis is demonstrable. (3) Symptoms do not recur and weight gain is normal for one month on a low-antigen formula. (4) Challenge with milk or soy formula, or other offending food antigens, reproduces symptoms. Powell also proposed a detailed method for oral food challenge (OFC) as a diagnostic test and criteria for positive responses. Briefly, when more than three of the following five criteria are positive, the challenge is considered positive[9]: (1) vomiting and/or diarrhea symptoms, (2) blood in the stool, (3) leukocytes in the stool, (4) eosinophils in the stool and (5) a change in the blood polymorphonuclear neutrophil count. Although OFC is the most conclusive diagnostic method, it has been associated with a risk of systemic reactions.[9-12,14,17,18,21] Therefore, OFC for diagnostic confirmation can be omitted when the clinical course is typical.[15] In recent years, the diagnostic criteria described by Sicherer have been more commonly used.[10] They are: (1) younger than 9 months of age at initial diagnosis, (2) repeated exposure to the incriminated food elicited diarrhea and/or repetitive vomiting within 24 hours without any other plausible cause for the symptoms, (3) there were no symptoms other than gastrointestinal symptoms elicited by the incriminated food and (4) removal of the offending protein from the diet resulted in resolution of the symptoms, and/or a standardized food challenge elicited diarrhea and/or vomiting within 24 hours after administration of the food.

EXAMINATIONS

The skin-prick test (SPT)[13,17] and specific IgE antibodies[13] for offending foods in the serum are negative in the majority of patients with FPIES at the time of diagnosis. However, some patients have detectable IgE[10,17,36,38] and positive SPT reactions[16,38,40] to offending foods. The symptoms in these patients tend to persist,[10,15] and those patients often develop IgE-mediated food allergy.[16,38,40,41] However, the role of such specific IgE antibodies in the pathogenesis of FPIES remains unclear.

The antigen-specific lymphocyte stimulation test (ALST) is a well-known method for investigating antigen-specific T-cell responses and theoretically should be suitable for diagnosis of FPIES that is thought to be cell-mediated. However, the usefulness of ALST for diagnosis of FPIES has been controversial.[42-45] We recently found that certain amounts of lipopolysaccharide (LPS) that contaminated commercially available cow's milk proteins used in previous reports are able to induce proliferative responses that are antigen-non-specific. In addition, the lymphoproliferative response to LPS was higher in neonates than in young children.[46] Therefore, ALST could be a helpful tool for diagnosis of FPIES if LPS-depleted cow's milk protein preparations are used.

Fogg reported the usefulness of the atopy patch test (APT) for initial diagnosis of FPIES, with 100% sensitivity, 71% specificity, 75% positive predictive value and 100% negative predictive value.[47] However, a more recent report found that APT does not predict outgrowing of FPIES (11.8% sensitivity, 85.7% specificity, 40% positive predictive value and 54.5% negative predictive value).[48] Further studies are needed to elucidate the usefulness of APT for initial diagnosis and prediction of outgrowing FPIES.

TREATMENT

The primary therapy for FPIES is to avoid the causal food antigens, just as for IgE-mediated food allergies. In infantile cases of FPIES caused by cow's milk, breastfeeding tends to be protective. Therefore, 1) exclusive breastfeeding is recommended when possible. However, a small number patients with FPIES react to cow's milk or soy protein passed through the breast milk.[25,36,37] Thus, some account should be taken of this possibility. 2) If exclusive breastfeeding is impossible, cow's milk should be replaced with other nutrition, such as a hydrolysate-based formula, soy-based formula or amino acid formula. Among those formulas, hydrolysate-based formula is preferable to soy-based formula, because it was reported that about half of FPIES patients are sensitive to both milk and soy.[10,17,47,49,50] However, recent reports indicate that the rates of coexisting milk and soy sensitivity are much lower than formerly thought.[13,38] 3) On the other hand, some patients tend to react even to hydrolysate-based formula.[51-53] For them, an amino acid formula is needed.

PATHOGENESIS

The histological findings of patients with FPIES have been reported to be uncharacteristic inflammation: edema, villous atrophy and cellular infiltration in the duodenum and jejunum.[54,55] However, TNF-α expression in the epithelial cells and mononuclear cells in the lamina propria was markedly increased in FPIES patients, especially those having villous atrophy.[55] In addition, TNF-α was highly secreted, antigen-specifically, by PBMC from patients with FPIES[46,56,57] and was also increased in the stool after milk challenge of patients with gastrointestinal milk allergy.[58,59] TNF-α is known to increase intestinal permeability.[57,60] Therefore, TNF-α could be involved in the pathogenesis of FPIES through alteration of intestinal permeability.

Thrombocytosis and leukocytosis after ingestion of offending foods are sometimes observed in patients with FPIES.[13,22] In addition, C-reactive protein (CRP) is often elevated in the sera from patients with GI allergy.[36,61,62] Both reactive thrombocytosis and elevation of CRP are known to be induced by IL-6. We recently found that IL-6 was highly produced, antigen-specifically, by PBMC from patients with GI allergy.[46] Thus, in addition to TNF-α, IL-6 may play some role in the pathogenesis of FPIES.

FOOD PROTEIN-INDUCED PROCTOCOLITIS (FPIP) (DIETARY PROTEIN-INDUCED COLITIS)

HISTORY

Cases of infants with rectal bleeding in the first few months of life have been reported since the 1950s.[63-67] However, in most cases no direct cause for rectal bleeding was able to be identified. In 1982, Lake *et al.* first suggested cow's milk protein passed through breast milk as a possible cause of rectal bleeding, which they experienced in a series of 6 infants who developed bloody diarrhea in the first month of life while being exclusively breast fed.[68] All 6 patients improved after being switched to hydrolyzed milk or soy-based formula, and the bloody diarrhea relapsed in all after being switched back to breast milk. In addition, elimination of cow's milk protein from the maternal diet led to tolerance of breast milk in 2 of 5 patients. Therefore, Lake *et al.* named those cases "dietary protein-induced colitis," meaning "food antigen-specific".[68] Sampson subsequently termed it "food protein-induced proctocolitis" (FPIP).[69]

CLINICAL FEATURES

Patients with FPIP typically develop grossly blood-streaked stool with mucus in the first few months of life. In contrast to FPIES, almost all patients with

FPIP develop no systemic symptoms and seem to be well except for the bloody stool. They have no growth delay or poor weight gain.[68,70-72] Mild anemia is seen in rare cases.[70,72-74] Many patients with FPIP are breast-fed, and the cause is thought to be mainly cow's milk protein passed through the breast milk.[68,71,73,75] Cow's milk and soy-based formulas are the major causative foods in the remaining cases.[74,76] Recently, FPIP have also been seen to manifest in childhood.[77] However, it remains unclear whether FPIP in childhood has the same pathogenesis as that in infants.

DIAGNOSIS

In 1982, Lake *et al.* observed a series of six infants who developed bloody diarrhea caused by cow's milk protein passed through breast milk and confirmed the antigen-specificity using both the antigen elimination test and OFC.[68] Subsequently, there were many reports of infants who developed bloody stool in the first few months of life and showed resolution of the symptoms after being switched to a hydrolyzed milk or soy-based formula.[73,74,76,78] Based on those reports, subsequent papers have assigned a clinical diagnosis of FPIP without OFC to apparently healthy infants with bloody stool and whose symptoms disappear on an elimination diet.

However, recent studies have questioned the accuracy of the elimination diet in diagnosis of the antigen-specificity of FPIP.[79-81] To elucidate the effect of a cow's milk elimination diet on the duration and severity of rectal bleeding in infants, Arvola *et al.*[79] randomly assigned infants with bloody stool to start a cow's milk elimination diet or to continue their current diet. As a result, the cow's milk elimination diet did not affect the duration or severity of rectal bleeding between the two groups. In addition, only 2 of 19 patients who started the cow's milk elimination diet experienced recurrence of gastrointestinal symptoms after reintroduction of cow's milk protein.[79] Jang *et al.* reported that 10 of 16 patients with a chief complaint of rectal bleeding showed resolution of symptoms without any dietary change, while the remaining 6 cases responded to a cow's milk elimination diet. However, only 2 of those 6 responders developed symptoms after OFC.[81]

These findings indicate that infants with bloody stool might include a small percentage of patients with FPIP that is actually caused in an antigen-specific manner. In support of this notion, Ohtsuka *et al.* reported 2 infants who had bloody stool on the first day of life, before initial feeding, but subsequently never developed bloody stool even with breast milk without maternal diet modification.[82] These facts suggest that there are some special enteral environments in early infancy that may result in antigen-nonspecific hemorrhage in the colon.

In that sense, patients with bloody stool whose antigen specificity was confirmed by both an elimination diet and OFC should be referred to as "true" FPIP. However, in this review we are going to treat patients with bloody stool as having FPIP even if they were diagnosed only by an elimination diet, without OFC. Thus, a certain proportion of FPIP patients may have antigen-nonspecific bloody stool, but in order to discuss FPIP, we must include these patients because only little studies other than Lake *et al.*'s confirmed antigen specificity in their subjects by OFC.

EXAMINATIONS

The endoscopic findings for FPIP are lymphonodular hyperplasia (LNH), with an oozing and edematous mucosal surface. The common histological features are LNH[70,71,78,83-86] and numerous eosinophils in the lamina propria.[70,71,73,74,76,78,83,86,87] Accordingly, apparently healthy infants with bloody stool and LNH and/or numerous eosinophils in the lamina propria were histologically diagnosed as FPIP and also called "allergic colitis".[73,74,76,78,83]

LNH in the colon has been reported to be associated with food allergy.[88-90] LNH can also be observed in other diseases, not just in FPIP.[88,89,91] In addition, Xanthakos reported several patients with LNH who had spontaneous resolution of rectal bleeding without any dietary change.[80] These findings suggest that LNH may be a self-limiting, age-related change, regardless of the antigen specificity.

A histological finding of eosinophil infiltration (6 cells/high power field (HPF)) in the lamina propria was thought to be a useful threshold for diagnosis of FPIP.[75,83] However, DeBrosse *et al.* recently described that eosinophils (mean 16-20 cells/HPF) were normally observed in the gastrointestinal tract of control children, especially in the colon.[92] In addition, Jang *et al.* reported that only 2 of 10 patients who exhibited marked eosinophil infiltration of the lamina propria (more than 6 cells/HPF) developed symptoms after OFC.[81]

Therefore, diagnosis of FPIP based on elimination tests, the endoscopic findings and the histological findings presents a risk of over-diagnosis. In this context, for accurate diagnosis of FPIP, OFC after an elimination diet should be recommended for apparently healthy patients with bloody stool.

TREATMENT

As in the case of other food allergies, elimination of causal food antigens is the gold-standard treatment for FPIP. Many patients with FPIP are breast-fed infants. Therefore, elimination of cow's milk from the maternal diet would be the first choice and an effective method.[71] However, some patients still react to breast milk even after the mother has strictly eliminated cow's milk from her diet.[68,78,93] For such patients, the infant's nutrition should be changed to a hydrolyzed milk or soy-based formula. However,

some patients respond even to hydrolyzed milk.[51,78,80] For them, an amino acid formula is needed.

PATHOGENESIS

Based on the endoscopic and pathological findings, activation of eosinophils and lymphocytes associated with LNH is thought to play an important role in the pathogenesis of FPIP. In support of this notion, Ohtsuka *et al.* recently showed that CCL11 (eotaxin-1) mRNA and CXCL13 mRNA were highly expressed in the large-intestine mucosa of infants with FPIP compared with control subjects.[94] However, the pathogenic mechanisms underlying induction of eosinophilic inflammation in the colon remain unclear.

INTESTINAL MICROBIOTA

Several investigator groups have reported an interesting finding that delayed maturation of the intestinal flora possibly causes rectal bleeding in infants.[79,95,96] In normal newborn babies, there is transient intestinal colonization by facultative anaerobes immediately after birth. Thereafter, obligate anaerobes such as *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Lactobacillus* species increase and establish colonization, with reduction of facultative anaerobe counts. However, the counts of obligate anaerobes, especially *Bifidobacterium*,[79,95] *Lactobacillus*,[79] *Clostridium leptum* group (*C. leptum) and Clostridium coccoides* group (*C. coccoides*),[96] were significantly lower in the feces of patients with FPIP than in healthy breast-fed infants. These findings suggest that there is delayed maturation of the intestinal flora in patients with FPIP.

In addition, Atarashi *et al.* revealed that a mixture of 46 strains of *Clostridium spp.*, including *C. leptum* and *C. coccoides*, promote regulatory T cell (Treg cells) accumulation and also affect their function in the colon of mice.[97] The fact that there are lower counts of *C. leptum* and *C. coccoides* in the feces of patients with FPIP suggests that impaired induction of Treg cells in the colon may be involved in the pathogenesis of FPIP. In support of this, Cseh *et al.* found that the ratio of Treg cells in PBMC was lower in patients with FPIP than in control subjects.[98] Moreover, *C. leptum* and *C. coccoides* also promote accumulation of IgA-positive cells in the colon[99] and induce IgA production through induction of Treg cells that produce TGF-β.[100] In support of this, secretory IgA concentrations in the feces tend to be lower in patients with FPIP than in control subjects.[96] These findings suggest that impaired induction of Treg cells and IgA (which contribute to homeostasis in the intestine) due to delayed maturation of the intestinal flora may be a cause of FPIP. In fact, children who outgrew their GI allergy had higher frequencies of circulating Treg cells after OFC compared with children with persistent GI allergy.[101]

However, it remains totally unknown how antigen-specific immune responses link to delayed maturation of the intestinal microbiota. Further investigation is needed to elucidate whether the underlying mechanisms are the same between patients with FPIP who really respond to food antigens specifically and those who do not.

FOOD PROTEIN-INDUCED ENTEROPATHY (ENTEROPATHY)

HISTORY

Patients with food protein-induced enteropathy (Enteropathy) were initially described by several investigators in the 1960s as malabsorption syndromes caused by cow's milk protein.[102-105] Subsequently, in the late 1970s, such patients were described as having "enteropathy induced by cow's milk".[106,107]

CLINICAL FEATURES

Patients with Enteropathy typically develop chronic diarrhea and show poor weight gain in the first several months of life.[108] Mild to moderate anemia and hypoproteinemia were seen in some patients with Enteropathy.[108] The clinical features of Enteropathy are similar to those of celiac disease that is associated with sensitivity to wheat protein. However, celiac disease is not included in this entity.

DIAGNOSIS

The diagnostic procedure for Enteropathy relies heavily on elimination tests and OFC. Jejunal biopsy is often helpful for diagnosis and follow-up.[108]

PATHOPHYSIOLOGY

The histological findings of patients with Enteropathy were well characterized in the 1970s to 1980s. In brief, jejunal biopsy specimens from patients with Enteropathy showed various degrees of villous atrophy with crypt hyperplasia,[109-111] increased numbers of intraepithelial lymphocytes,[108,112] and increased numbers of lymphocytes, plasma cells and eosinophils in the lamina propria.[113]

Chung *et al.* reported that extracellular deposition of major basic protein (MBP), which is an eosinophil granule protein, in the lamina propria was significantly higher in patients with Enteropathy than in control subjects. In addition, extracellular deposition of MBP correlated significantly with the severity of mucosal villous atrophy.[114] These findings suggest that MBP released by eosinophils plays an important role in the pathogenesis of Enteropathy by inducing mucosal damage that leads to the villous atrophy.

However, patients with Enteropathy have rarely been reported since 2000.[115]

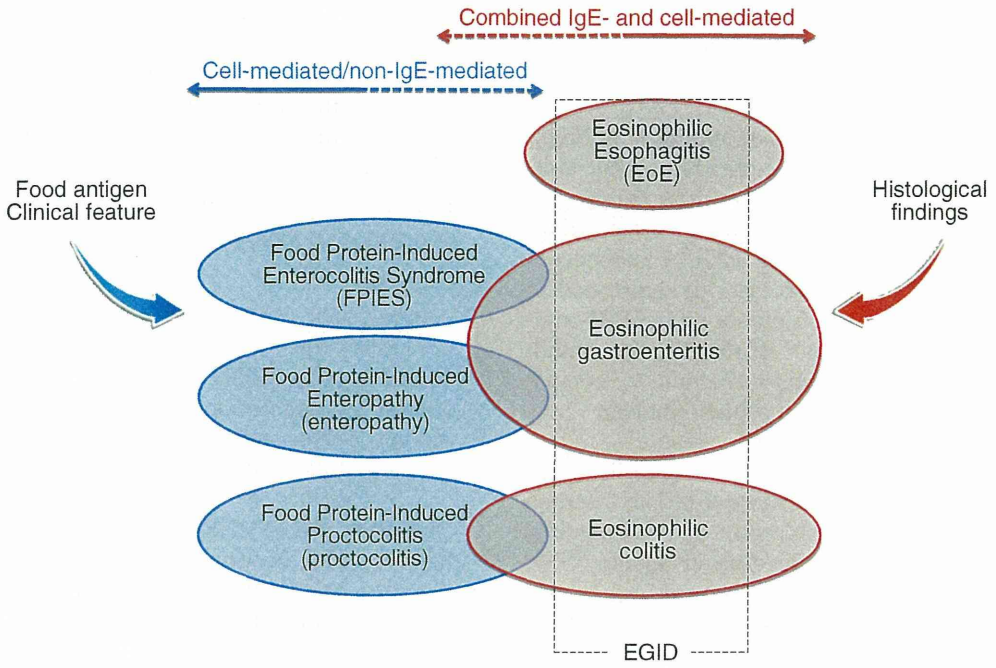

Fig. 2 Clinical and histological characterization of GI allergies. From the standpoint of an allergist, GI allergies (FPIES, FPIP and Enterocolitis) are diseases characterized by antigen-specific immune responses (left-hand side). On the other hand, from the standpoint of a gastroenterologist, gastrointestinal diseases with eosinophil infiltration are called eosinophilic esophagitis, gastroenteritis and colitis (right-hand side). The disease pathogenesis, especially how much these entities may overlap, warrants further investigation.

CURRENT TOPICS AND FUTURE PERSPECTIVES

FOUR DISTINCT CLINICAL SUBTYPES IN NON-IgE-MEDIATED FOOD ALLERGIES IN INFANTS

We recently tried to classify infants with gastrointestinal symptoms by using cluster analysis of their clinical and laboratory findings.[36] We found that these patients can be classified into 4 distinct subtypes according to the presence or absence of vomiting and bloody stool. In brief, patients who had vomiting with or without bloody stool were likely to be diagnosed as having FPIES. Patients who had only bloody stool were likely to be diagnosed as having proctocolitis. Patients who had neither vomiting nor bloody stool were likely to be diagnosed as having enteropathy. In all four clusters, oral food challenge tests showed remarkably high reproducibility of the symptoms found at the initial presentation, even though the oral challenge tests were performed several months after the initial presentation. This observation suggests that the upper or lower gastrointestinal tract-specific hypersensitivity and perhaps the responsible immune cells remain in the same part of the gastrointestinal tract even after several months' remission.

ROLE OF IgE ANTIBODIES IN GI ALLERGY

Some patients with non-IgE-mediated GI allergies have detectable levels of food-specific IgE antibodies.[10,17,38] In the abovementioned study,[36] a positive milk-specific IgE antibody titer was observed in 37% of the patients, with no statistically significant differences among any of the 4 subtypes. Interestingly, almost all symptoms at initial presentation as well as in OFC began to manifest at more than two hours after ingestion of the offending food, but 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 fully explains the gastrointestinal symptoms nor rules out a diagnosis of non-IgE-mediated GI allergy in infants.

In contrast, EGID was classified as a combined IgE- and cell-mediated disease because many patients with EGID have detectable food-specific IgE antibodies.[7] However, the roles of IgE antibodies in the pathogenesis of EGID remain unclear.

The precise roles of IgE antibodies in GI allergy and EGID warrant further investigation.

CYTOKINE SECRETION PROFILES IN GI ALLERGY

We recently determined—for the first time—the

antigen-specific cytokine secretion profiles of PBMC from infants with non-IgE-mediated GI allergy using extensively LPS-depleted milk antigens and 89 blood samples originating from all over Japan through the Japanese Research Group for Neonatal, Infantile Allergic Disorders.[46] We found significantly high concentrations of TH2 cytokines, but not TH1 or TH17 cytokines, in the culture supernatants of those PBMC. The pathogenesis of non-IgE-mediated gastrointestinal allergy had been thought to be "non-TH 2" cell-mediated because IgE antibody is usually undetectable. However, our results strongly indicate that the pathogenesis is, in fact, "TH2" cell-mediated. In particular, high levels of IL-13 and TNF-α may play critical roles in intestinal epithelial cell damage and eosinophil infiltration, presumably through activation of the tumor necrosis factor-like weak inducer of apoptosis-fibroblast growth factor-inducible molecule 14 (TWEAK-Fn14) axis.[116] However, it remains unclear why these patients do not produce antigen-specific IgE antibodies. It may be due to reduced expression of the IL-4 receptor alpha chain and reduced IL-4-induced signaling in neonatal B cells. Further studies are needed to elucidate this issue.[117]

DISEASE ENTITIES OF GI ALLERGY AND EGID

The definitions and diagnostic criteria for GI allergy and EGID were drawn up from different perspectives. Non-IgE mediated diseases such as FPIES, FPIP and Enteropathy are diagnosed mainly on the basis of the clinical manifestation, as described earlier. On the other hand, combined IgE- and cell-mediated diseases such as EGID including eosinophilic gastroenteritis, eosinophilic colitis and eosinophilic esophagitis (EoE) are diagnosed mainly on the basis of a single histological finding, that is, inappropriate accumulation of eosinophils in the gastrointestinal tract.[118] However, many investigators reported that such accumulation is often observed even in patients with non-IgE-mediated GI allergy. Therefore, there must be some overlap and similar pathogenic mechanisms between non-IgE-mediated GI allergy and EGID.[7] In support of this, we recently found that—along with such inflammatory cytokines as TNF-α and IL-6—Th2 cytokines, including IL-3, IL-5 and IL-13, but not Th1 cytokine or Th17 cytokine, were significantly produced *in vitro* by milk protein-stimulated PBMCs from infant patients with GI allergy compared with control subjects.[46] These findings suggest that antigen-specific Th2-type immune responses underlie the pathogenesis of non-IgE-mediated GI-allergy (Fig. 1).

The incidence of each GI allergy is relatively low in comparison to that of IgE-mediated food allergy.[38,62] Therefore, the diagnostic and classification criteria depend heavily on small numbers of cases experienced by various investigators and were drawn up from their different standpoints, for example, as a pediatrician, an allergist, a neonatologist and a gastroenterologist. The low prevalence and the different standpoints may have strongly impacted on the different diagnostic criteria for GI allergy and EGID (Fig. 2). To resolve this matter and to obtain a complete view of GI allergy, we need to conduct a multicenter study with cooperation among pediatricians, allergists, neonatologists and gastroenterologists.

Fortunately, we have constructed a nation-wide database using an internet online system and have obtained data for over 500 patients with GI allergy, thanks to the cooperation of doctors all over Japan. That database has started to generate valuable results, step by step.[36,46] We hope to carry out further investigations that will fully elucidate the pathogenesis of GI allergy.

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Elevated Plasma Cytokines in Japanese Patients with Eosinophilic Esophagitis and Gastroenteritis

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Key Words
Elevated plasma cytokines · Eosinophilic esophagitis · Eosinophilic gastroenteritis

Abstract
Background/Aim: The role of Th2-type cytokines in development of eosinophilic esophagitis (EoE) has been largely revealed, whereas research on the pathogenesis of eosinophilic gastroenteritis (EGE) has not been widely performed. We investigated the possible involvement of Th2-type cytokines in EGE by measuring plasma cytokine concentrations in patients with EGE as well as those with EoE. ***Methods:*** 18 patients with EoE, 18 with EGE, and 30 normal volunteers were enrolled in the study. Plasma concentrations of five cytokines (thymic stromal lymphopoietin, IL-5, IL-13, IL-15, and eotaxin-3) were measured using Milliplex® assays. Clinical characteristics of the patients and plasma cytokine levels were then compared. ***Results:*** Higher proportions of patients with EoE and those with EGE showed elevated plasma concentrations of IL-5 and IL-15 as compared to the normal controls. There was also a positive correlation between IL-5 and IL-15, and also with blood eosinophil count. The plasma concentrations of these cytokines tended to be higher in cases with EGE than in those with EoE, though there were overlaps in cytokine levels among the patients and controls. ***Conclusion:*** Similar increases in plasma IL-5 and IL-15 were observed in patients with EoE and those with EGE.

Eosinophilic esophagitis (EoE) is a disease characterized by dense infiltration of eosinophils in esophageal mucosa [1], and its incidence and prevalence are increasing in Western countries [2–4]. Based on both animal and human studies, the pathogenesis of EoE is considered to be as follows. First, food or environmental allergens stimulate esophageal epithelial cells, Th2 lymphocytes, dendritic cells, and augment thymic stromal lymphopoietin (TSLP) secretion. Stimulated Th2 lymphocytes produce IL-5 and IL-13, and dendritic cells produce IL-15. Next, IL-5 stimulates proliferation of eosinophilic leukocyte progenitor cells, and IL-13 and IL-15 increase eotaxin-3 production by esophageal epithelial cells. Eotaxin-3 is a potent chemokine that facilitates the trafficking of eosinophils from peripheral blood to esophageal epithelium, where they along with the help of mast cells cause chronic inflammation and fibrosis by secreting various types of granular proteins and cytokines such as TGF-β

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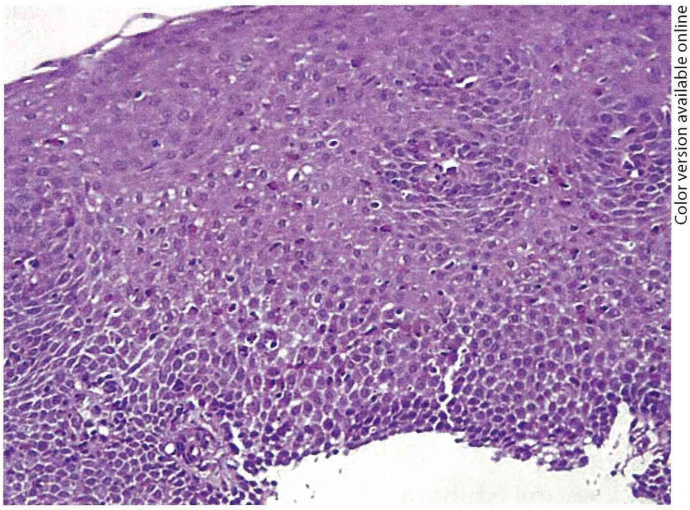

Fig. 1. Photomicrograph of an esophageal mucosal biopsy specimen from a typical patient with EoE. There are many eosinophils infiltrating in the epithelial layer of the esophagus. The number of eosinophils infiltrating in the epithelium is over 20 in one high-power microscopic field. ×400.

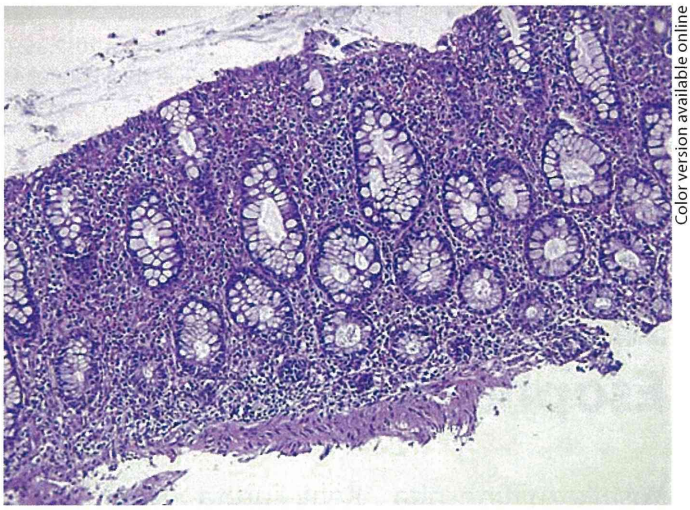

Fig. 2. Photomicrograph of a colonic mucosal biopsy specimen from a typical patient with EGE. There are many eosinophils infiltrating in the lamina propria of colonic mucosa. The eosinophil is the predominant inflammatory cell in the colonic mucosa. The number of eosinophils infiltrating in the mucosa is over 20 in one high-power microscopic field. ×400.

[5–10]. Patients with EoE have been reported to have higher concentrations of these cytokines in peripheral blood than normal individuals [8, 11, 12]. Thus, TSLP, IL-5, IL-13, IL-15, and eotaxin-3 are considered to be important cytokines for development of EoE in both experimental animals and humans. Eosinophilic gastroenteritis (EGE) is a disease related to chronic dense infiltration of eosinophils in gastrointestinal mucosa and may have a similar pathogenetic mechanism as EoE, though the precise details are not yet clarified [13, 14].

Diagnosis of EoE and EGE is primarily based on histological confirmation of dense eosinophil infiltration in esophageal and gastrointestinal mucosa. However, such eosinophilic infiltration is not specific to EoE/EGE, as other diseases are also known to display such infiltration in esophagogastrointestinal mucosa. Histological confirmation of mucosal eosinophilic infiltration in patients with EGE is not easy when lesions are present in the small intestine. In that regard, peripheral blood eosinophil count and eosinophil granular protein concentrations have been investigated as possible markers for diagnosis of EoE [8, 11, 12].

We speculated that EGE has a pathogenetic mechanism similar to that of EoE, and that affected patients have elevated peripheral blood concentrations of TSLP, IL-5, IL-13, IL-15, and eotaxin-3. In the present study, plasma concentrations of these cytokines were determined in patients with EoE and EGE, as well as in normal individuals to examine their possible role in the pathogenesis of EGE as well as for use in non-invasive diagnosis.

Materials and Methods

Cases

Eighteen patients with EoE and 18 with EGE were enrolled. The mean age of those with EoE was 50.9 ± 4.1 years and 11 (61%) were male. Ten reported symptoms of dysphagia, 4 of heartburn, 3 of chest pain, and 1 of back pain. All EoE cases had histologically confirmed esophageal epithelial eosinophil infiltration >15/HPF and satisfied the diagnosis recommendation proposed by the American Gastroenterological Association [1] (fig. 1). The mean age of the patients with EGE was 50.6 ± 4.7 years and 9 (50%) were male. Nine of those reported abdominal pain and 7 diarrhea. All of the patients with EGE had histologically confirmed gastrointestinal mucosal dense eosinophil infiltration (fig. 2). Diseases other than EGE that causes dense eosinophil infiltration in the gastrointestinal tract such as parasitic infections and inflammatory bowel diseases were carefully ruled out from EGE and excluded from this study. EGE patients with food allergy were not excluded from this study, since food allergy is considered to be a possible pathogenetic factor of EGE. Six EGE cases included in this study had food allergy (3 cases to seafood, 2 cases to eggs, and 1 case to nuts). In addition, 30 normal volunteers (mean age 40.8 ± 2.4 years, 22 males) served as controls.

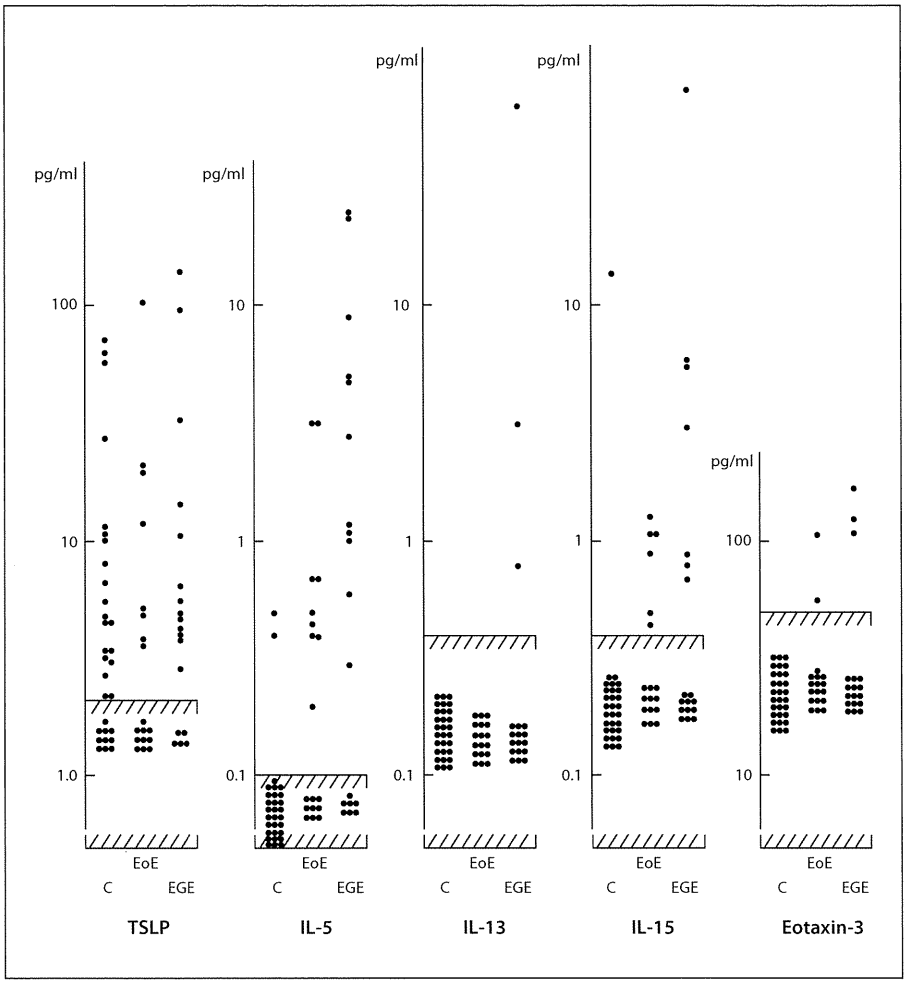

Fig. 3. Plasma concentrations of TSLP, IL-5, IL-13, IL-15, and eotaxin-3 in patients with EoE and EGE, and in normal controls (C). Vertical lines show logarithm scales. Each dot represents a single case. Shaded zones show ranges under minimal detection concentrations.

Plasma was collected before the start of glucocorticoid administration from all of the patients. In 1 with EoE and 2 with EGE, plasma samples were also collected during prednisolone treatment.

Plasma Concentrations of TSLP, IL-5, IL-13, IL-15, Eotaxin-3, and Interferon-γ

The plasma samples were separated and frozen at –30°C before measurements of cytokines. IL-5, IL-13, and IL-15 concentrations were determined using a Milliplex® Human Cytokine/Chemokine Kit (Millipore Corp., St. Charles, Mo., USA), while those of TSLP and eotaxin-3/CCL26 were determined with a Milliplex Human Cytokine/Chemokine Panel II kit (Millipore Corp.). Interferon-γ (IFN-γ) was measured by an EIA kit (Medgenix IFN-γ EASIA kit; BioSource Europe SA, Belgium). The minimum detected concentrations (mDCs) of TSLP, IL-5, IL-13, IL-15, eotaxin-3 and IFN-γ in the present assays were 2.1, 0.1, 0.4, 0.4, 50 pg/ml and 0.1 IU/ml respectively. All assays were done in duplicate and the mean value was used as the plasma concentration in each sample (fig. 3, 4a–c).

The protocol of the study was approved by the ethical committee of Shimane University School of Medicine.

Statistical Analysis

To examine differences between the EoE/EGE patients and controls, a χ^2 test and t test were used, as appropriate. Pearson's product-moment correlation coefficient was used to determine the correlation between peripheral blood eosinophil count and cytokine concentrations. Values are shown as mean ± SE. A p value <0.05 was considered to be statistically significant.

Results

Plasma concentrations of TSLP were over the mDC in 20 of the 30 (67%) controls and ranged from not detectable (ND) to 70.7 pg/ml. In addition, that concentration was over the mDC in 8 of 18 (44%) patients with EoE (range: ND to 100.2 pg/ml) and 13 of 18 (72%) with EGE (ND to 145 pg/ml). Thus, there was a significant overlap in TSLP concentrations between patients in both groups and the normal controls (fig. 1). IL-5 concentrations were

over the mDC in 7% of the normal controls, and in 50 and 61% of the EoE and EGE patients, respectively, which showed a significant difference between the controls and patients (EoE, p = 0.0005; EGE, p = 0.00004). There was no significant difference with regard to age, gender, or history of allergic diseases between patients with and without detectable plasma IL-5. As for IL-13, only 3 patients with EGE had that cytokine detected in plasma, while none with EOE and none of the controls had an IL-13 concentration >0.4 pg/ml. On the other hand, a significantly higher number of patients had detectable IL-15 in plasma as compared to the controls (EoE, p = 0.004; EGE, p = 0.001). There were no significant differences with regard to age, gender, or history of allergic diseases in patients with and without detectable plasma IL-15. None of the controls had detectable eotaxin-3, while that was detected in only 2 and 3 of the EoE and EGE patients, respectively (fig. 1). In 18 cases with EGE, there was no significant difference in plasma cytokine concentrations between 6 EGE cases with food allergy and 12 EGE cases without food allergy.

Only 1 (6%) of 18 patients with EoE and 6 (33%) of 18 with EGE had abnormally elevated CRP concentrations, with mean values of 0.2 ± 0.1 and 1.2 ± 0.6 mg/dl, respectively. There was no positive co-relation between the CRP level and the plasma cytokine levels we measured in this study. 5 with EoE (28%) and 13 with EGE (72%) had elevated blood eosinophils over 600/μl, while the mean eosinophil count was 399 ± 100/μl in patients with EoE and 2,348 ± 1,261/μl in those with EGE. Thus, patients with EGE showed higher CRP and blood eosinophil levels as compared to those with EoE.

When the correlations among plasma concentrations of TSLP, IL-5, IL-15, and blood eosinophil count were examined, there were significantly positive correlations seen among IL-5, IL-15, and eosinophil levels (fig. 2), while there was no correlation between those of TSLP and eosinophils. Correlations among IL-13, eotaxin-3, and blood eosinophil count were not examined, as only a small number of samples had detectable plasma IL-13 and eotaxin-3 levels.

Three patients, 1 with EoE and 2 with EGE, were treated with glucocorticoid administrations, and their CRP, blood eosinophil count, and cytokine levels were repeatedly measured. Only TSLP concentrations were within a detectable range in these 3 cases. In the patient with EoE, the only detectable plasma cytokine was TSLP, which decreased along with the decline of CRP and eosinophil count. In 1 patient with EGE, TSLP did not change in spite of a remarkable decline in CRP and eosinophil count, while in the other with EGE, TSLP and other in-

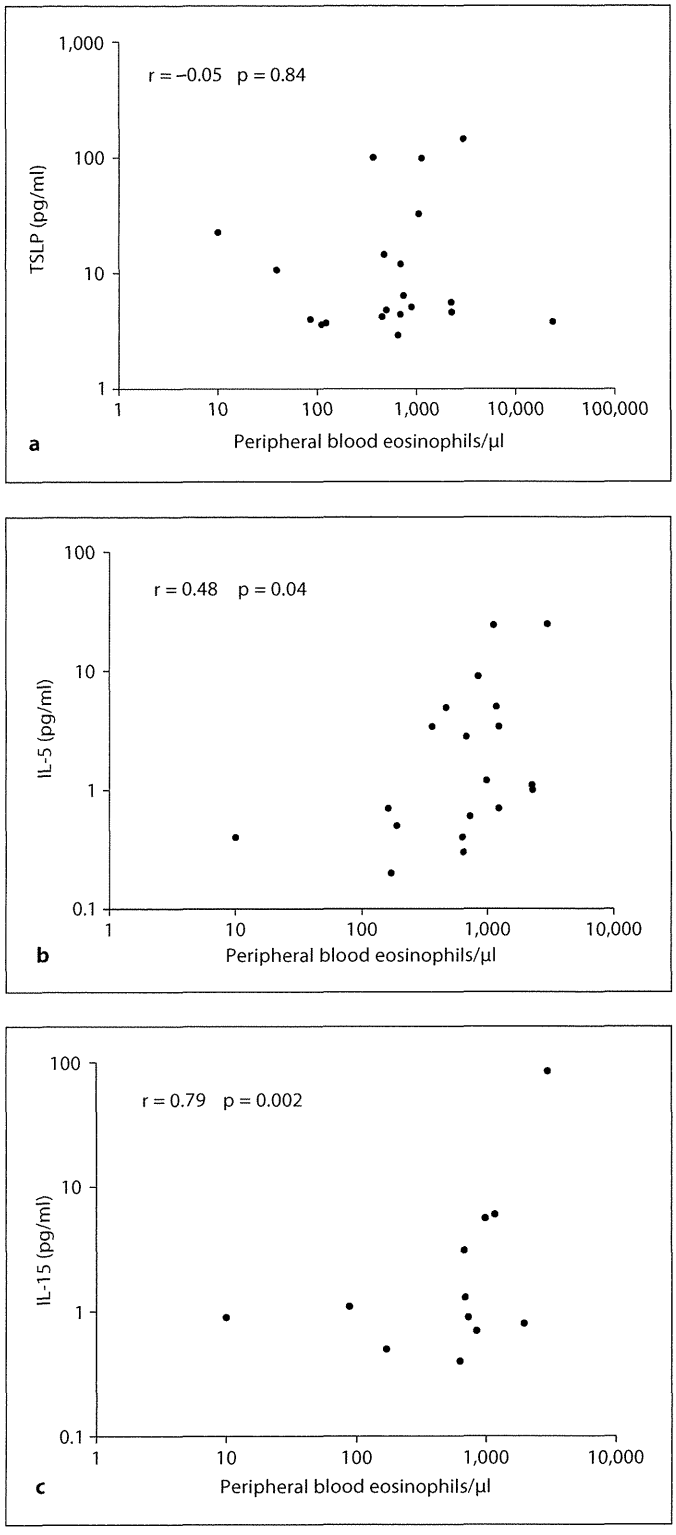

Fig. 4. Correlations between blood eosinophil count and plasma TSLP (**a**), IL-5 (**b**), and IL-15 (**c**) levels. There were positive correlations among IL-5 and IL-15 levels, and blood eosinophil count. All the EoE and EGE patients with cytokines over the mDC were included in the analyses.

flammatory markers did not change with glucocorticoid administration.

IFN-γ was measured only in cases with EoE or with EGE and was not measured in plasma of normal controls because of the limitation of the samples. According to the manufacturer, the normal value of plasma IFN-γ concentration was reported to be 0.2 ± 0.3 (SD) IU/ml. In patients with EoE or EGE, all the patients except 1 case with EGE showed plasma IFN-γ under the minimal detection level of the assay kit (0.1 IU/ml).

Discussion

The incidence rates of allergic diseases including atopic dermatitis, bronchial asthma, and pollinosis are reported to be increasing in developed countries. During development of these diseases, a dominant Th2 response over that of Th1 is considered to be important [15]. The limited chance of chronic bacterial infection during childhood, e.g. because of the decreasing rate of *Helicobacter pylori* infection, in developed countries is considered to be a reason for the increasing prevalence of allergic diseases in Th2-dominant responders [16].

EoE and EGE are allergic digestive diseases characterized by chronic inflammation with dense mucosal infiltration of eosinophils. Many patients with EGE have been reported in Asian countries, with increasing numbers with EoE reported in Western countries [2, 17, 18]. We have previously reported that, in the routine endoscopic study, a EoE patient was found in approximately 0.02% of the endoscopy investigated Japanese cases [19]. In Western countries, on the other hand, EoE patients were reported to be found in 0.4–0.5% of the endoscopy investigated cases [4, 20]. In addition, the prevalence of EoE in Western countries was reported to be over 10 cases in 100,000 general population [21]. Approximately 30 patients with EGE were found annually in our survey conducted in Japan from 2004 to 2009 [22]. For confirmation of diagnosis and grading of disease severity, histopathological evaluations of the involved lesions are important, thus endoscopic examinations along with biopsies of targeted mucosal tissues are inevitable. Repeated endoscopic examinations are difficult and inconvenient for the patient. Therefore, inflammatory markers such as CRP and peripheral eosinophil count have been used to support a diagnosis of EoE or EGE, and to evaluate disease activity before and during treatment in a non-invasive manner. However, these markers are not adequately sensitive and only some patients show increased values [3, 17, 23].

In patients who develop EoE, augmented local production of TSLP, IL-5, IL-13, IL-15, and eotaxin-3 has been reported at both mRNA and protein levels [9, 12, 24–26].

When locally produced, these cytokines are released into systemic circulation and their plasma concentrations are expected to be elevated. Indeed, recent findings have suggested elevated plasma concentrations of eotaxin-3, IL-5, IL-13, and other inflammatory cytokines in patients with EoE [8, 11, 12, 27, 28], thus indicating roles for these cytokines in the pathomechanism of EoE, as shown in experimental results [29]. If EGE causes a similar increase in plasma cytokines, its pathomechanism may be considered similar to that of EoE. In the present study, the plasma concentrations of five different cytokines reported be related to development of EoE were determined and compared in EGE and EoE patients, and normal volunteers.

As reported previously, IL-5 and IL-15 concentrations were significantly elevated in both EoE and EGE patients. These similar cytokine responses suggest a similar role for these cytokines in EoE and EGE. There was also a large overlap in the plasma levels of IL-5 and IL-15 observed in the patients and controls, though those levels were significantly elevated in the patients. Therefore, measurement of these cytokines has limited value for diagnosis of EoE and EGE, as described in consensus recommendations [30]. In addition, plasma TSLP and IL-13 levels were nearly the same between the EoE patients and controls, while there was a small but significant increase in IL-13 in patients with EGE.

Plasma IL-5, IL-13, IL-15, and eotaxin-3 in patients with EGE were higher than in those with EoE. In addition, patients with EGE had a higher peripheral blood eosinophil count than those with EoE. There was a significantly positive correlation between plasma IL-5 and IL-15, and between IL-5 and peripheral eosinophil count. Therefore, IL-5 and IL-15 may have direct or indirect roles in eosinophilia.

In 3 of the patients, plasma cytokine levels were measured before and during glucocorticoid treatment. Contrary to our expectation, plasma cytokines did not change remarkably during treatment in those patients, while CRP and eosinophil count decreased in 2 of 3 cases. Those results do not support measurements of plasma cytokines to evaluate the response of patients to glucocorticoid treatment.

There are some limitations to this study. First, the number of patients in each group may not be large enough to obtain conclusive results. Although patients with EoE and EGE are limited in Japan, and it is difficult to collect information regarding them, a large-scale nationwide