Increased CD69 Expression on Peripheral Eosinophils from Patients with Food Protein-Induced Enterocolitis Syndrome

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Abstract

Background: Food protein-induced enterocolitis syndrome (FPIES) is an uncommon, non-IgE-mediated food allergy. We recently described a significant increase in fecal eosinophil-derived neurotoxin (EDN) after ingestion of the causative food. However, little is known about the activation status of circulating eosinophils in patients with an acute FPIES reaction.

Methods: Surface CD69 expression was assessed by flow cytometry on peripheral eosinophils from five patients with FPIES before and after ingestion of the causative food. Fecal EDN was measured by an enzyme-linked immunosorbent assay.

Results: No eosinophil activation was observed before ingestion, however, a significant increase in CD69 expression on eosinophils after an acute FIPES reaction was demonstrated in all of the patients. There was no significant change in absolute eosinophil counts in the peripheral blood. The levels of fecal EDN increased on the day after ingestion of the causative food in all patients.

Conclusion: These results suggest that circulating eosinophils as well as eosinophils in the intestinal mucosal tissue are activated in acute FPIES reactions and might be associated with systemic immune events in FPIES.

Introduction

Non-IgE-mediated gastrointestinal food allergy includes food protein-induced enterocolitis syndrome (FPIES), food protein-induced proctocolitis, and food protein induced enteropathy [1]. FPIES is a rare disorder that usually occurs in young infants and is characterized by severe gastrointestinal tract symptoms [2]. Profuse vomiting, lethargy, and pallor typically start within 1 to 3 h of causative food ingestion, and diarrhea begins within 5 to 8 h. The most common causative foods are cow's milk and soy. FPIES is usually diagnosed based on medical history, response to an elimination diet, and an oral food challenge [3].

Recent evidence suggest that antigen-specific T cells, eosinophils, and cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-10, and transforming growth factor (TGF)-β, are involved in the pathophysiology of FPIES [1-3]. We have recently reported elevation of fecal eosinophil-derived neurotoxin (EDN) in infants with FPIES [4]. This finding may support the role of eosinophils in gastrointestinal inflammation in FPIES, however, the nature of circulating eosinophils in acute FPIES reactions remains to be elucidated. In this report, we describe the expression of activation marker CD69 on circulating eosinophils from patients with FPIES and discuss the systemic events in acute FPIES reactions.

Materials and Methods

We studied five Japanese patients with FPIES. Patients 1, 2, 3, and 4 have been described in our previous report as patients P1, P3, P4, and P5, respectively [4]. The diagnosis of FPIES was based on the previously established criteria: 1) repeated exposure to the incriminated food elicits repetitive vomiting and/or diarrhea within 24 h, without any other cause for the symptoms, 2) symptoms are limited to the gastrointestinal tract, and 3) removal of the offending protein from the diet results in resolution of the symptoms and/or a food challenge elicits vomiting and/or diarrhea within 24 h after administration of the food [5-7]. After making a diagnosis of suspected FPIES, trigger foods were eliminated from the diet of all patients.

We performed an oral food challenge test in four patients (patients 1 to 4) in the hospital according to both the guidelines of Powell et al. [8] and the Japanese guidelines for food allergy [9], as described previously [4]. Briefly, patients were given either the same amount of trigger food that caused the reactions or up to 0.6 g protein/kg body weight. The diagnostic food challenge test was not given to patient 5, who had more than three episodes of the typical reactions.

Peripheral blood samples were drawn from patients both before the food challenge test and up to 6 hours after an acute FPIES reaction. Expression of activation marker CD69 on the cell surface of eosinophils was evaluated by flow cytometry, as described previously [10]. Fecal samples were collected from patients before and after the food challenges. In patient 5, samples were collected after accidental ingestion of the causative food. If the patients did not spontaneously defecate on the day after the ingestion, glycerin enemas were given. Fecal samples were collected at each defecation during the next 1 to 2 days. Control samples were obtained from 12 age-matched healthy

infants. Feces were processed, and the levels of fecal EDN were measured using a commercially available enzyme-linked immunosorbent assay kit (MBL, Nagoya, Japan), as previously described [4]. Analysis of differences among the groups was performed using the Student's t-test, and differences with p-values less than 0.05 were considered significant. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

Results

Table 1 presents the clinical and laboratory data of the patients. The mean age of onset was 9.3 ± 8.8 mo, and the mean time to diagnosis was 3.2 ± 2.6 mo. The common symptoms were vomiting (5/5), lethargy (3/5), and diarrhea (2/5). All of the patients developed symptoms 2 h after eating. All 4 of the patients who underwent the food challenge test exhibited typical FPIES reactions, which did not differ from their medical history derived from the accidental ingestion of the causative food. The time between the most recent reaction and the food challenge test was 1.6 ± 1.3 mo (Table 1). After elimination of the causative food, no episodes of FPIES were noted in any patient. No patients experienced IgE-mediated acute symptoms, such as urticaria and wheezing.

Although a change in the total blood polymorphonuclear leukocyte count greater than $3500/\mu l$ is one of Powell's criteria for a positive challenge, only patient 1 showed leukocytosis with neutrophilia (fig. 1a). C-reactive protein levels and absolute eosinophil counts were not increased in all patients. In contrast and consistent with our previous

report [4], a significant increase in fecal EDN on the day after ingestion of the causative food was found in all patients (mean 26,670 ng/ml; fig. 1b). The median time to maximum concentration of fecal EDN was 30 h.

To evaluate the activation status of peripheral eosinophils, we compared CD69 expression before and after acute FPIES reactions (fig. 2). The mean time to blood sampling after an acute FPIES reaction was 3.0 ± 2.8 h. No eosinophil activation was observed before ingestion of the causative food, however, a significant increase in CD69 expression after ingestion was found in all patients.

Discussion

A FPIES reaction is a cell-mediated response to food proteins. Indeed, it has been reported that T cells proliferate in response to food antigens in patients with FPIES [11]. However, there is some controversy over the usefulness of the T cell proliferative response for diagnosis, because the stimulation index is not consistently different from that in pediatric control subjects [12]. T cells activated by food antigens could release proinflammatory cytokines, such as TNF-α, that are known to increase intestinal permeability and mediate local intestinal inflammation. Studies on the small intestinal mucosa from FPIES patients demonstrated the presence of TNF-α released by lamina propria T cells and decreased activity of TGF-β1, implicating the changes in these molecules in the pathogenesis of FPIES [13]. A recent study described predominant skewing of antigen-specific T-cell responses toward Th2 in FPIES, in which peripheral blood mononuclear cells from patients produced significantly more TNF-α, IL-3, IL-5,

and IL-13 compared with that produced by control subjects [14]. In addition to these T-cell mediated events, involvement of innate cells in local inflammation in the gastrointestinal tract has been observed. Smears of fecal mucus from positive challenge patients revealed the presence of neutrophils, eosinophils, and lymphocytes [15]. We and others have recently reported increased levels of fecal EDN in patients with FPIES [4,16]. It is also noted that an acute FPIES reaction results in an increase in the number of peripheral neutrophils and platelets [5,7,15]. The former is included in the diagnostic criteria proposed by Powell [8]. On the other hand, circulating eosinophil counts are decreased after a positive food challenge [17]. However, the activation status of these innate cells in the gastrointestinal tracts as well as in the peripheral blood is not fully understood.

Our patients exhibited a significant increase in fecal EDN after ingestion of the causative food. However, eosinophils are a normal component of intestinal mucosal tissue. Therefore, it has remained unclear whether the elevation of fecal EDN was derived from increased permeability of the mucosa or from eosinophil activation and degranulation at the reaction sites or both. It is difficult to perform endoscopic examination and biopsy in FPIES infants during an acute FIPES reaction. There is no animal model for FPIES. In this study, we demonstrated activation of circulating eosinophils after an acute FPIES reaction. Although we do not know whether such activation is an initial event triggered by food antigens or reflects a secondary immune response following intense systemic reactions, it seems reasonable to assume that eosinophils in the intestinal mucosal tissue are also activated, resulting in degranulation of various inflammatory mediators, including EDN. Similar results were obtained from a

study of eosinophils from patients with bronchial asthma, where CD69 was expressed both on locally activated lung eosinophils and on circulating eosinophils after an *in vivo* challenge with inhalation allergens [18,19]. In addition, severe cases of atopic dermatitis exhibited systemic allergic inflammation, resulting in expansion of activated eosinophils in the peripheral blood [10]. Accordingly, the induction of CD69 on circulating eosinophils could be a more general consequence of severe eosinophil-associated allergic inflammation. Nevertheless, since this study cohort is small, larger studies will be required to confirm our observations. Further investigation will be also necessary to assess the role of eosinophil activation and immune mechanisms linking antigen-specific T cell responses and innate cell activation in FPIES.

In summary, our results demonstrate induction of the activation marker CD69 on circulating eosinophils after an acute FPIES reaction and point to an additional component of the systemic events in this disease.

Acknowledgments

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Table 1. Patient characteristics.

Patients	1	2	3	4	5
Age at onset (mo)	10	7	7	6	26
Age at diagnosis (mo) [a]	16	13	11	8	26
Age at OFC (mo)	16	13	11	8	NA
Time between the most recent reaction and OFC (mo)	3.4	1.6	0.4	0.8	NA
Sex	F	M	M	F	F
Trigger food	Fish [b]	Egg	Wheat	Rice	Egg
Symptoms at home	Vomiting Lethargy	Vomiting Lethargy Diarrhea	Vomiting lethargy	Vomiting diarrhea	Vomiting Pallor
Symptoms during OFC	Vomiting Lethargy	Vomiting Lethargy	Vomiting Pallor	Vomiting diarrhea	NA
Time to symptoms (h)	2	2	2	2	2
Total IgE (IU/mL)	5	5	28	22	90
Specific IgE (U$_A$/mL)	fish <0.35	egg <0.35	egg 9.88 wheat <0.35 gluten <0.35	egg 0.87 rice <0.35	egg 0.80

[a] Age of confirmed diagnosis by the challenge test (patients 1 to 4) or by clinical assessment (patient 5). [b] Sebastes alutus. OFC, oral food challenge; NA, not applicable.

1 **Figure Legend**

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4 **Fig. 1.** Changes in laboratory findings. (**a**) Leukocyte counts and C-reactive protein

5 (CRP) levels before and after ingestion of the causative food. WBC, white blood cells.

6 (**b**) Fecal eosinophil-derived neurotoxin (EDN) was measured before and after ingestion

7 of the causative food. The maximum concentration of fecal EDN on the next day after

8 ingestion is shown. Shaded areas represent the ranges of the normal values. Analysis of

9 differences among the groups was performed using the Student's t-test, and differences

10 with p-values less than 0.05 were considered significant. ns, not significant. *$p < 0.05$;

11 **$p < 0.01$; ***$p < 0.001$.

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14 **Fig. 2.** Analysis of CD69 expression on circulating eosinophils. (**a**) Surface CD69

15 expression was analyzed on eosinophils that were defined as CD16-negative cells in a

16 granulocyte region by flow cytometry. Open and solid histograms indicate CD69

17 expression before and after ingestion of the causative food, respectively. (**b**) Mean

18 fluorescent intensity (MFI) of CD69 expression. Error bars represent the standard

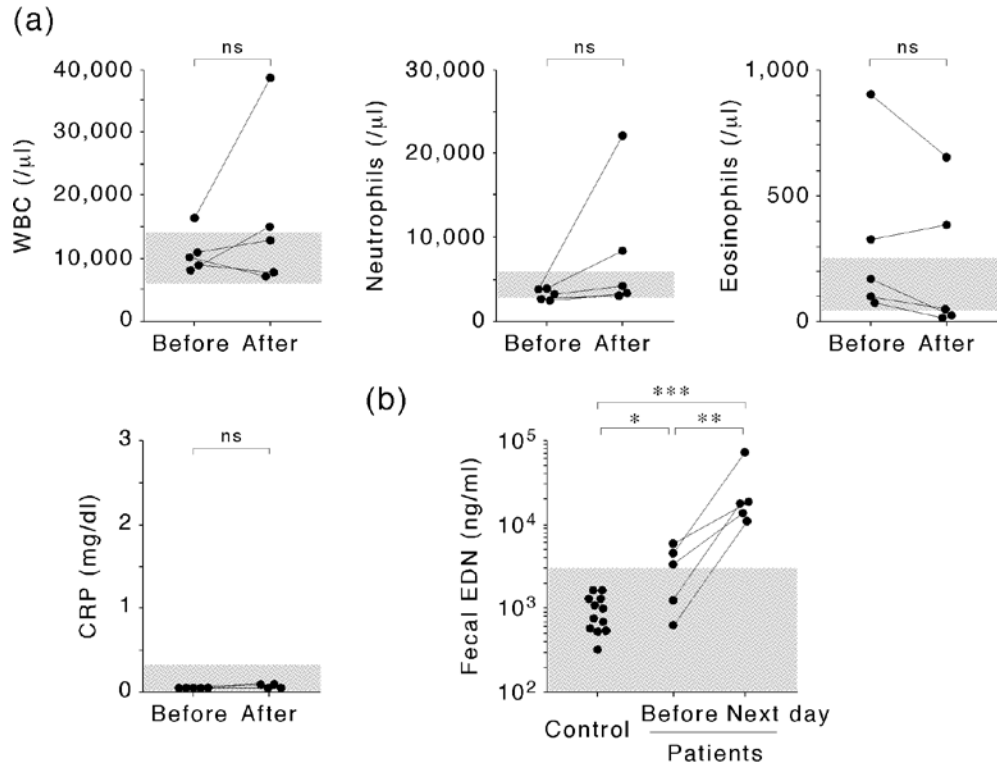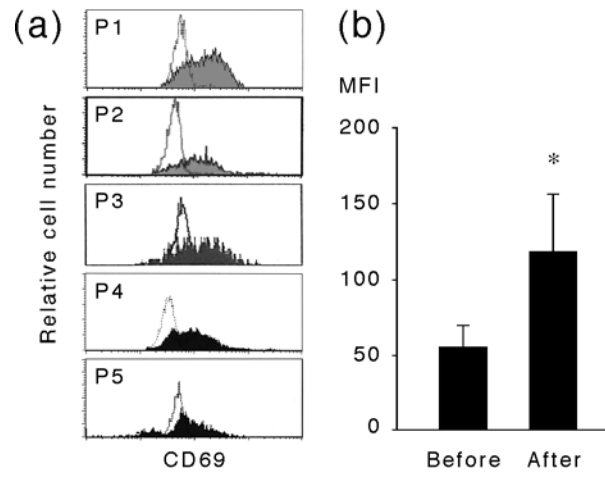19 deviation. P, patient. *$p < 0.05$.

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21   **Figure 1**

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27 **Figure 2**

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