Cytokine profile after oral food challenge in infants with food protein-induced enterocolitis syndrome

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Introduction

Food protein-induced enterocolitis syndrome (FPIES) is a relatively rare disease characterized by gastrointestinal (GI) symptoms such as vomiting and diarrhea without any reactions in the skin or the respiratory tract.1,2 It usually develops in neonates or infants after ingesting cow milk (CM) formula. The pathology of the disease is the inflammation of the intestine.3,4 Since food-specific IgE antibody (sIgE) is seldom detected in patients with FPIES,5 it is classified as a subtype of non-IgE-mediated gastrointestinal food allergy.6 By contrast, the proliferation of peripheral blood lymphocytes stimulated with causative food proteins is shown to be increased in most cases.6,7,8,9 Thus, cell-mediated hypersensitivity is supposed to play an important role in the pathogenesis of the disease.

However, little is known about the pathophysiology of FPIES. So far, some laboratory and experimental findings that might reflect the pathophysiology of FPIES have been reported. Increases in the absolute neutrophil count (ANC)9 and the serum level of C-reactive protein (CRP)10 were induced in oral food challenge (OFC).
Correspondingly, an increased production of tumor necrosis factor-alpha (TNF-α) in the intestinal tissue was demonstrated. On the other hand, an increased production of Th2 or Th2-inducing cytokines has also been demonstrated in the serum of the patients and in the culture supernatant of peripheral blood mononuclear cells stimulated with CM proteins. These findings might be related to eosinophilia in the peripheral blood or the intestinal tissue.

However, the role of those cytokines in the development of GI symptoms or abnormal laboratory findings has not yet been clarified. In order to find a clue about the role of cytokines, we analyzed the alteration of serum cytokine levels at OFCs in patients with FPIES.

**Methods**

**Subjects**

Four patients with FPIES, who were referred to our institute between January 1, 2013, and May 31, 2016, were enrolled in this study (Table 1). The causative food was CM formula in all cases. Diagnosis of FPIES was made according to the following criteria defined by the Japanese Guidelines for Food Allergy 2014: 1) development of GI symptoms without symptoms typically seen in IgE-mediated food allergy after the ingestion of causative food, 2) disappearance of symptoms at OFC with CM formula, and 4) exclusion of other diseases such as infections, surgical problems, and so on. The reproducibility of GI symptoms by accidental re-exposure to the CM formula was considered equivalent to a positive response during planned OFC. These criteria correspond to that utilized in a recent large-scale study. If a patient received OFC twice, the first and second OFC are represented by the patient number. This study was approved by the ethics committee of Shizuoka Children's Hospital and the Faculty of Medicine, University of Toyama. Informed consent was obtained from the parents before the subjects were included in the study and data were collected from the medical records.

**OFC**

A planned OFC was performed according to a stepwise daily incremental protocol, which was made to reduce the incidence of severe reactions. The volume of the CM formula on the first day was 5 mL/kg (up to 50 mL), which was increased the next day to the volume usually ingested by the subject if no symptoms were seen. In cases where symptoms at onset or previous OFC were very severe, the volume of the CM formula on the first day was reduced to 1 mL/kg (up to 5 mL), and was doubled daily to the volume usually ingested by the subject if OFC was uneventful. If any symptoms developed, no more CM formula was given thereafter.

The result of OFC is judged positive if any GI symptoms (vomiting, diarrhea, or bloody stool) were induced.

**Measurement and grading of cytokines**

Blood samples were obtained before challenge of the CM formula and daily thereafter until the end of the OFC. If any symptoms were observed, an extra blood sample was collected six hours after the last ingestion of CM formula to examine the early responses.

Serum levels of the following cytokines were measured by flow cytometry with Diaclone Diaplex Immunoassay Kit (Gen-ProbeDiaclone SAS, Besançon, France) according to manufacturer’s instructions: IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, interferongamma (IFN-γ), TNF-α, and IL-1β. The serum level of IL-5 was also measured by flow cytometry with human Th1/Th2 cytokine kit (Cytometric Bead Array, Becton Dickinson Biosciences, CA, USA).

The changes in the serum cytokine level after the challenge of the CM formula were graded by comparison with the pre-challenge level and expressed as follows: mild increase (+), more than 50% to less than 100%; moderate increase (++) more than 100% to less than 500%; and remarkable increase (+++), more than 500%. In order to calculate, cytokine levels lower than the lower limit of the sensitivity of the kit were substituted arbitrarily by the lower limit of the sensitivity.

**Measurement and grading of CRP level, and absolute number of neutrophils and eosinophils**

Serum CRP level, ANC, and absolute eosinophil count (AEC) were measured at the same time points as the measurement of cytokine profile.

The increase in the serum CRP level, ANC, and AEC was graded into 3 stages and expressed as follows: CRP: mild increase (+), less than 0.5 mg/dL; moderate increase (++), from 0.5 mg/dL to less than 2.0 mg/dL; of CRP: remarkable increase (+++), 2.0 mg/dL or higher; ANC: mild increase (+), less than 1000/μL; moderate increase (++), from 1000/μL to less than 3500/μL; remarkable increase, more than 3500/μL; AEC: mild increase, less than 50/μL; moderate increase, from 50 μL to less than 200/μL; remarkable increase, more than 200/μL.

**Other laboratory examinations**

CM-sIgE level was measured using the ImmunoCAP system (Thermo Fisher Scientific Inc., Tokyo, Japan). CM-specific lymphocyte proliferation (Allergen-specific lymphocyte stimulation test

**Table 1**

Demographic and clinical profiles of subjects.

<table>
<thead>
<tr>
<th>No</th>
<th>Sex</th>
<th>GA (w)</th>
<th>BW (kg)</th>
<th>Age</th>
<th>Symptom</th>
<th>CM-sIgE</th>
<th>CRP</th>
<th>ALST</th>
<th>At onset</th>
<th>At OFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>M</td>
<td>37</td>
<td>3.1</td>
<td>1 d</td>
<td>V, Fe</td>
<td>&lt;0.35</td>
<td>9.32</td>
<td>+</td>
<td>P1-I</td>
<td>19 m</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>P1-II</td>
<td>30 m</td>
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<td></td>
<td></td>
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<td></td>
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<td>12 m</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P3</td>
<td>1 m</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P4</td>
<td>7 m</td>
</tr>
</tbody>
</table>

Demographic data of subjects at the onset and during OFC were shown in addition to the clinical and laboratory findings.

ALST: allergen specific lymphocyte stimulation test; B, bloody stool; BW, birth weight; CM, cow milk; CRP, C-reactive protein [mg/dL]; d, day; D, diarrhea; De, dehydration; F, female; Fe, fever; GA: gestational age (week); m, month; M, male; N, negative; No, patient number; No-O, patient number at OFC; OFC, oral food challenge; OFC-N, number of times of OFC; P, positive; sIgE, specific IgE (kU/L); V, vomiting; W, body weight.
(ALST) for CM was commercially measured by BML Inc. (Saitama, Japan) according to the previously reported method. The concentrations of CM proteins used for stimulation of lymphocytes were as follows: αs-casein, 50 μg/mL; β-casein, 400 μg/mL; κ-casein, 100 μg/mL; β-lactoglobulin, 200 μg/mL; and lactoferrin, 100 μg/mL. The concentration of endotoxin was managed to be lower than 100 pg/mL. The positivity in the test was determined based on the upper limit of the normal range.

Results

Subject profiles

Three of 4 subjects were male (Table 1). The median gestational age was 38 weeks (range 37–39 weeks), and the median birth weight was 3.1 kg (range 2.6–3.2 kg). The age of onset ranged from 1 day to 1 month after birth. CM-sIgE was not detected in 3 of 4 subjects, while all 4 subjects showed positive results in ALST for CM. Serum level of CRP was increased in 3 out of 4 patients.

At the time of OFC, age ranged from 1 to 30 months (median 15 months) and body weight ranged from 4.0 to 14.7 kg (median 9.0 kg). Four OFCs were positive and two were negative. CM-sIgE was negative in 4 of 6 OFCs. In addition to GI symptoms, fever was seen in 3 positive OFCs (P1-I, P2-I, P3).

Laboratory findings

An increase in the serum CRP level was seen in all 4 positive OFCs (O1–4): 3 patients with moderate increases (0.5 to <2.0 mg/dL) (O1–3) and 1 patient with a remarkable increase (>2.0 mg/dL) (O4) (Table 2, 3). An increase in the serum CRP level was not seen in 2 negative OFCs.

An increase in ANC was also seen in all 4 positive OFCs: 2 moderate increases (1000 to <3500/μL) (O2, 4) and 2 remarkable increases (>3500/μL) (O1, 3). As for AEC, a moderate increase was seen in a positive OFC (O2), and a remarkable increase was observed in a negative OFC (O5) (Table 2, 3).

Cytokines increased in positive OFCs

Serum levels of IL-2, IL-5 and IL-8 were found to rise after 4 positive OFCs where elevations of the serum CRP level and ANC were also caused (Table 2, 3). Regarding IL-5, mild (O5) and remarkable (O6) increases in the serum level were also seen after 2 negative OFCs.

Serum levels of IL-10 increased in 2 positive OFCs (O1, 2) performed consecutively on the same subject (P1). Increases in serum level of IFN-γ, IL-6, TNF-α, and IL-12 were observed in a positive OFC (O4) with a remarkable increase in the serum CRP level.

Table 2

Summary of the symptoms, laboratory findings, cytokine profiles.

<table>
<thead>
<tr>
<th>OFC No</th>
<th>Pt No-O</th>
<th>Symptom</th>
<th>CRP</th>
<th>ANC</th>
<th>AEC</th>
<th>IL-2</th>
<th>IL-5</th>
<th>IL-8</th>
<th>IL-10</th>
<th>IFN-γ</th>
<th>IL-6</th>
<th>TNF-α</th>
<th>IL-12</th>
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<tbody>
<tr>
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<td>P1-I</td>
<td>V, Fe</td>
<td>P</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>O2</td>
<td>P1-II</td>
<td>V</td>
<td>P</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>P2-I</td>
<td>D, Fe</td>
<td>P</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>P3</td>
<td>D, Fe</td>
<td>P</td>
<td>++</td>
<td>+</td>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>O5</td>
<td>P2-II</td>
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<td>N</td>
<td>--</td>
<td>+</td>
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<td>++</td>
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</tr>
<tr>
<td>O6</td>
<td>P4</td>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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</tr>
</tbody>
</table>

The changes in laboratory data and serum cytokine levels are summarized. Data of IL-4, IL-17A, and IL-1β is excluded because their responses are poor or irregular. The changes in the serum cytokine level, the serum CRP level, ANC and AEC were graded as follows: cytokine: mild increase (+), more than 50% to less than 100%; moderate increase (++) more than 100% to less than 500%; remarkable increase (+++) more than 500%; CRP: mild increase (+), less than 0.5 mg/dL; moderate increase (++) from 0.5 mg/dL to less than 2.0 mg/dL; remarkable increase (+++) 2.0 mg/dL or higher; ANC: mild increase (+), less than 1000/μL; moderate increase (++) from 1000/μL to less than 3500/μL; remarkable increase, more than 3500/μL; AEC: mild increase, less than 50/μL; moderate increase, from 50/μL to less than 200/μL; remarkable increase, more than 200/μL.

Table 3

Time course of serum cytokine levels and laboratory data.

<table>
<thead>
<tr>
<th>OFC No</th>
<th>Pt No-O</th>
<th>Symptom</th>
<th>Time (h)</th>
<th>Laboratory data</th>
<th>Cytokine-A (pg/mL)</th>
<th>Cytokine-B (pg/mL)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CRP, ANC, AEC</td>
<td>IL-2, IL-5, IL-8, IL-10, IFN-γ, IL-6, TNF-α, IL-12</td>
<td>IL-4, IL-17A, IL-1β</td>
</tr>
<tr>
<td>O1</td>
<td>P1-I</td>
<td>V, Fe</td>
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<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>25.7</td>
<td>1.6</td>
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<td></td>
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<td></td>
<td>6</td>
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<td></td>
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<td>0.5</td>
<td>25.7</td>
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<td>&gt;40.0</td>
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<td>P2-I</td>
<td>D, Fe</td>
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<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
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<td></td>
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<td>25.7</td>
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<td>P3</td>
<td>D, Fe</td>
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<td>&lt;0.2</td>
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<tr>
<td></td>
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<td>0.1</td>
<td>&gt;40.0</td>
</tr>
<tr>
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<td>&lt;0.2</td>
<td>&lt;0.2</td>
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<tr>
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<td>0.5</td>
<td>25.7</td>
<td>2.7</td>
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<td></td>
<td></td>
<td>6</td>
<td>20.5</td>
<td>0.1</td>
<td>&gt;40.0</td>
</tr>
</tbody>
</table>

Alteration of laboratory data and serum cytokine levels during OFC were shown. The change of ANC and AEC was shown as the differences from data before OFC (μL). Cytokine-A, cytokines increased after ingestion of food; Cytokine-B, cytokines poorly or irregularly increased after ingestion of food.

ANC, absolute neutrophil count (μL); AEC, absolute eosinophil count (μL); B, bloody stool; CRP, C-reactive protein (mg/dL); D, diarrhea; Fe, fever; OFC, oral food challenge; Pt No-O, patient number at OFC; V, vomiting.
There were no clear specific correlations between cytokines and GI symptoms except that vomiting was seen at 2 OFCs in which the serum IL-10 level rose (O1,2).

**Cytokines increased poorly or irregularly in positive OFCs**

Changes in serum levels of IL-4 and IL-1β were irregular (Table 3). Serum levels of IL-4 were increased in some positive OFCs (O3, 4), but decreased in other case (O1). Although serum IL-1β could be detected in a positive OFC (O1), it lowered after ingestion of food. On the contrary, the serum level of IL-1β rose after ingestion of food in a negative OFC (O6). Serum levels of IL-17A did not show any increase.

**Time course of serum cytokine levels**

Serum levels of IL-2 and IL-8 reached their peak values simultaneously 6 h after ingestion of CM formula and returned to pre-stimulation levels within 24 h in 3 positive OFCs (O1-3) (Table 3, Fig. 1). In these patients, moderate increases in the serum CRP level were also observed 24 h after ingestion of food. IL-10 showed a similar time course to IL-2 and IL-8 in 2 positive OFCs (O1, 2). By contrast, an increase in the serum level of IL-2, IL-5, IL-6, IL-8, IL-12, and IFN-γ persisted for more than 24 h in a positive OFC (O4) (Fig. 2). In this OFC, serum levels of IL-2, IL-6, IL-8, and TNF-α were first to reach their peak levels, being followed by IFN-γ and IL-12.

Increases in the serum level of IL-5 persisted for more than 24 h in 3 out of 4 positive OFCs (O1, 2, 4) and 2 negative OFCs (O5, 6) (Table 3).

**Discussion**

Although cell-mediated hypersensitivity is assumed to be a major immunological mechanism of FPIES, little is known about its pathogenesis or pathophysiology. A cytokine profile may be a useful tool to elucidate the function and role of allergen-specific
lymphocytes in the pathogenesis of FPIES. TNF-α was shown to be expressed in the intestine.\textsuperscript{11} Many cytokines are reported to be produced by peripheral blood lymphocytes when stimulated with CM proteins.\textsuperscript{12} However, the role of each cytokine in the development of GI symptoms has not yet been confirmed. In this study, we attempted to elucidate the pattern of cytokine increase in the serum of FPIES patients challenged with the CM formula. The results revealed that the serum levels of IL-2, IL-5, and IL-8 were consistently increased in all positive OFCs in patients with FPIES.

IL-2 is a representative Th1 cytokine and activates T cells, B cells, and monocytes/macrophages.\textsuperscript{16} The fact that serum level of IL-2 increased in all positive OFCs suggests that IL-2 from Th1 cells may play a significant role in the pathogenesis of FPIES. The pathology of FPIES is presented as the inflammation of the intestine.\textsuperscript{1,3} Although chronic inflammatory bowel diseases, such as Crohn’s disease and ulcerative colitis, are also caused by inflammation of the intestine, the serum cytokine level of IL-17A, but not IL-2, is shown to be increased.\textsuperscript{17} This indicates the difference in the pathophysiology between FPIES and chronic inflammatory bowel diseases.

IL-5 is a representative Th2 cytokine that enhances the proliferation of eosinophils and promotes the development of eosinophilic inflammation in allergic disorders.\textsuperscript{20} Serum levels of IL-5 were also increased in all positive OFCs in the present study. However, an increase in the serum IL-5 level was also observed in negative OFCs. This raises a question about its role in the development of GI symptoms. Production of IL-5 might increase irrespective of the development of GI symptoms in some infants after ingestion of food. From this point of view, it is interesting that AEC in the peripheral blood increases in young healthy infants according to an increase in the feeding amounts.\textsuperscript{21}

Another surprising finding is that an increase in AEC in the peripheral blood was seldom observed in an OFC in which the serum IL-5 level increased. In contrast to ANC, AEC decreased in 3 of 4 positive OFCs. This may suggest that eosinophils cannot participate as major effector cells in the development of FPIES. However, further studies are needed to elucidate the role of IL-5 and eosinophils not only in the pathophysiology of FPIES, but also in the intestinal physiology of healthy infants, as well as in former FPIES patients who acquired tolerance.

An increase in the serum IL-8 levels was also seen in all positive OFCs. IL-8 is a well-known chemoattractant for neutrophils.\textsuperscript{22} Administration of IL-8 is demonstrated to increase ANC in the peripheral blood by mobilizing neutrophils from the bone marrow.\textsuperscript{23} Indeed, moderate to remarkable increases in ANC were observed in positive OFCs, in which the serum level of IL-8 was increased. Although Powell showed a remarkable increase in ANC during OFCs in FPIES patients,\textsuperscript{9} the mechanism is not yet known. This study suggests that IL-8 may play an important role in an increase in ANC during OFC.

We reported fever and an increase in the serum CRP levels in many patients with FPIES at OFC.\textsuperscript{10} In this study, moderate to remarkable increases in the serum CRP level were seen in all positive OFCs. Fever was seen in 3 of 4 positive OFCs. Serum levels of IL-2 and IL-8 were commonly increased in positive OFCs. IL-2 is well-known to induce fever and an increase in the serum CRP level in many patients treated with IL-2 for cancer.\textsuperscript{24} IL-8 is also shown to induce fever and the synthesis of CRP.\textsuperscript{25,26} Thus, these two cytokines may act as main factors inducing fever and a moderate increase in the serum CRP level in addition to the GI symptoms in FPIES patients.

In this study, serum levels of cytokines were not measured 6 h after ingestion of food in negative OFCs. Therefore, it could not be concluded whether serum levels of IL-2 and IL-8 changed or remained the same in negative OFCs. However, considering that these cytokines could elevate the production of CRP, the lack of an increase in the serum CRP level 24 h after ingestion of food suggests that they were secreted only in a smaller amount, if at all, in negative OFCs than in positive OFCs.

Some patients with FPIES showed a remarkable increase in the serum CRP level concurrent with GI symptoms and fever.\textsuperscript{27} These patients are described here as septicemia-like FPIES because of the similarity to severe neonatal infection or sepsis in symptoms and laboratory findings. In this study, not only IL-2 and IL-8, but also IFN-γ, TNF-α, IL-5, and IL-12 were found to be increased in an OFC showing septicemia-like symptoms and laboratory findings (O4). These proinflammatory cytokines are known to induce fever and to enhance the production of CRP.\textsuperscript{28,29} Serum levels of these cytokines are shown to be elevated in infants with sepsis.\textsuperscript{30,31} This result elucidates the similarity of the cytokine profile between septicemia-like FPIES and true sepsis. Since none of these cytokines were elevated in FPIES patients lacking a remarkable increase in serum CRP levels, they seem to be closely related to a remarkable increase in serum CRP levels.

**Acknowledgements**

This study was partly supported by grants from the Shizuoka Prefectural Hospital Organization.

**Conflict of interest**

The authors have no conflict of interest to declare.

**Authors’ contributions**

MK designed the study and wrote the manuscript. YI contributed to data collection and measured cytokines. MS, HM, and TM contributed to data collection. YA and SS provided critique for revision. All authors have read and approved the final manuscript.

**References**