Detection of Pro- and Anti-Inflammatory Cytokines in Stools of Patients with Inflammatory Bowel Disease

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Background: Cytokines play a predominant role in immune and inflammatory reactions in inflammatory bowel disease. Any cytokine that is produced locally as a result of gut inflammation may leak into the bowel lumen and appear in the stools. We examined the usefulness of determining cytokine profiles in the stools of patients with ulcerative colitis or Crohn’s disease. Methods: Cytokine concentrations in stool extracts were measured in 36 patients with ulcerative colitis, 32 patients with Crohn’s disease, 9 controls with inflammatory disease, and 18 normal controls by means of enzyme-linked immunosorbent assays. Results: Stool concentrations of interleukin-1\textsubscript{B} and interleukin-1 receptor antagonist in patients with active inflammatory bowel disease increased significantly and correlated with various inflammatory factors and stool concentrations of polymorphonuclear cell elastase. The ratio of interleukin-1 receptor antagonist to interleukin-1\textsubscript{B} in active disease was reduced significantly compared with that in inactive disease or in normal controls. Paired analysis showed a decrease in tumor necrosis factor-\textalpha and interleukin-1\textsubscript{B} and interleukin-1 receptor antagonist and an increase in interleukin-4 and interleukin-10 concentrations after the resolution of disease exacerbation. Conclusions: Measurement of cytokines in stools may be a useful and noninvasive means of understanding pathophysiology and clinical monitoring in inflammatory bowel disease.

Key words: Cytokine; inflammatory bowel disease; stool

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Although complete understanding of the role of cytokines in the pathophysiology of inflammatory bowel disease (IBD) has not been achieved, it is increasingly evident that these molecules in aggregate play a predominant role in immune and inflammatory reactions in this disorder (1–3). Whereas the concentration of cytokines in the intestine itself is important for the characterization of cytokine profiles in IBD (4–8), it is of course not suitable for practical use. It is also not known whether cytokines present in the peripheral circulation mirror intestinal inflammation. Therefore, we asked whether the stools of IBD patients contain increased amounts of cytokines and, if so, whether measurement of stool cytokines would be a reliable and noninvasive means of understanding the disease status in the gut. Further, preliminary reports have described significant amelioration of disease activity in patients with Crohn’s disease (CD) after systemic administration of anti-tumor necrosis factor (TNF)-\textalpha monoclonal antibodies (9) and in patients with ulcerative colitis (UC) after intracolonic administration of interleukin (IL)-10 (10). Therefore, the monitoring of cytokine profiles may be more relevant for evaluating the efficacy of immunotherapy. However, except for two reports on a pediatric population showing a significant increase in TNF\textalpha\textsubscript{2} (11) and IL-6 (12) during active disease, little is known about cytokine profiles in the stools of patients with IBD.

In the present study we identified and quantitated cytokines with pro- and anti-inflammatory properties in stools of IBD patients, using specific and sensitive enzyme-linked immunosorbent assays (ELISAs), and examined how the cytokines were related to clinical variables.

MATERIALS AND METHODS

Ethics

This project was performed in accordance with the Helsinki Declaration and was approved by the local medical ethics committee. Informed consent was obtained from every patient.

Subjects

Thirty-six patients with UC and 32 patients with CD were investigated. The diagnoses were based on characteristic clinical, endoscopic, radiologic, and histologic features.

Patients with UC. There were 23 men and 13 women with a median age of 32 years (range, 17–65 years). In terms of disease distribution, 19 patients had pancolitis, eight patients had left colon involvement, and 9 patients had disease limited to the rectum. Disease activity in each patient was analyzed in accordance with the criteria of Truelove & Witts (13). Nineteen patients had active disease (5, mild; 12, moderate;
2, severe) and 17 patients had inactive disease. At the time of study 13 patients had taken sulphasalazine only (5 active, 8 inactive); 7 patients, both corticosteroid and sulphasalazine (2 active, 5 inactive); 2 patients, corticosteroid only (2 active, 0 inactive); and 14 patients, no specific treatment (10 active, 4 inactive). Whenever possible, endoscopic findings were graded from 1 to 4 in accordance with the severity of inflammation, using the criteria of Matts (14).

Patients with CD. There were 20 men and 12 women, with a median age of 34 years (range, 17–67 years). In 14 patients the disease affected both the ileum and the colon, in 5 patients the colon only, and in 13 patients the ileum only. Disease activity was assessed on the basis of the score of the International Organization for the Study of Inflammatory Bowel disease (IOIBD) (15). A score of 1 or less was defined as corresponding to inactive disease, and a score of 2 or more corresponded to more active disease. In this group 18 patients had active and 14 patients inactive disease. Ten patients had taken sulphasalazine only (5 active, 5 inactive); 8 patients, both corticosteroid and sulphasalazine (4 active, 4 inactive); and 14 patients, no specific treatment (9 active, 5 inactive).

Control subjects. Eighteen healthy, age-matched subjects served as normal controls. Nine patients with other colitides (seven infectious and two ischemic colitis) were examined as inflammatory disease controls.

Sampling

Patients were instructed to defecate directly into a polystyrene container (diameter, 11.5 cm; depth, 7.5 cm). The stool extraction procedure for cytokine measurement has been
described previously (16, 17). In brief, stool was weighed, diluted 1:2 in phosphate-buffered saline (pH 7.2) containing soy trypsin inhibitor (1 mg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and phenylmethylsulfonyl fluoride (1 mg/ml; Wako Pure Chemical Industries, Ltd.) and centrifuged at 10,000 g at 4°C for 15 min. Supernatants were collected and kept frozen at −80°C until use. A blood sample was also obtained to determine various laboratory variables.

**Determination of stool samples**

Cytokines were measured in the stools, using sensitive ELISAs for IL-1β, TNFα (detection limit, <0.1 pg/ml for IL-1β and <0.18 pg/ml for TNFα; R&D Systems Inc., Minneapolis, Minn., USA), IL-1 receptor antagonist (IL-1ra) (<22.0 pg/ml, Amersham International plc, Biotrak, UK), IL-4 (<2.1 pg/ml, SRL Inc., Tokyo, Japan) (18), and IL-10 (<2.0 pg/ml, Sumitomo Metal Bio-Science Inc., Kanagawa, Japan) (19). A stool marker for disease activity, polymorphonuclear cell elastase, was determined with ELISA (<11.0 µg/l, Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan) (20).

**Validity testing**

The reliability of stool cytokine measurement was tested by making the following observations: a) The recovery rate from stool extraction was determined by mixing known quantities of cytokines with stools before grinding. Recovery of cytokine concentration in stool samples obtained from the same individual on different days (within 3 days) was always followed the standard curve. b) Sample titration gave detectable levels of cytokines that followed the standard curve. c) The variation of cytokine concentration in stool samples obtained from the same individual on different days (within 3 days) was <12.0% (n = 5).

**Determination of blood samples**

Routine clinical laboratory methods were used for additional assessment of acute-phase indicators, including the leukocyte count and erythrocyte sedimentation rate. Serum C-reactive protein concentrations (normal range <350 ng/ml) were measured with laser nephelometry (NA latex CRP kit; Hoechst Japan, Tokyo, Japan).

**Statistical analysis**

The means of multiple groups were compared by using Bonferroni’s t test after analysis of variance. Student’s t, Kruskal–Wallis, and Spearman rank correlation were also used for statistical analyses. Differences were considered statistically significant for any P < 0.05.

**RESULTS**

Individual stool cytokine concentrations are shown in Fig. 1. Cytokines were detected in every subject. Stool concentrations of IL-1β and IL-1ra were significantly higher in patients with active IBD than in patients with inactive disease and normal controls. There was no significant difference in IL-1β.

**Table 1. Correlation coefficients and significance of differences between stool cytokine concentrations and other laboratory variables in patients with inflammatory bowel disease**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
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</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
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<tr>
<td>IL-1ra</td>
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<tr>
<td>TNFα</td>
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<tr>
<td>C-reactive protein</td>
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<tr>
<td>1-h ESR</td>
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<tr>
<td>PME</td>
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<tr>
<td>C-reactive protein</td>
<td></td>
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</tbody>
</table>

**IL = interleukin; TNF = tumor necrosis factor; ESR = erythrocyte sedimentation rate; PME = polymorphonuclear cell elastase; IL-1ra = IL-1 receptor antagonist. Figures in parentheses are numbers of patients.**

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<thead>
<tr>
<th>Cytokine</th>
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<th>Crohn’s disease</th>
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</thead>
<tbody>
<tr>
<td>IL-1β</td>
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<tr>
<td>IL-1ra</td>
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<tr>
<td>TNFα</td>
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<td>C-reactive protein</td>
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<td>1-h ESR</td>
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<tr>
<td>PME</td>
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<tr>
<td>C-reactive protein</td>
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</tbody>
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**IL = interleukin; TNF = tumor necrosis factor; ESR = erythrocyte sedimentation rate; PME = polymorphonuclear cell elastase; IL-1ra = IL-1 receptor antagonist. Figures in parentheses are numbers of patients.**
and IL-1ra concentrations between active UC and CD. The ratio of IL-1ra to IL-1β in patients with active IBD was significantly lower than that in inactive disease or in normal controls. Stool concentrations of IL-4 and IL-10 in active disease were comparable to those of patients with inactive disease or controls.

There was significant correlation of stool IL-1β and IL-1ra with laboratory variables or stool concentrations of polymorphonuclear cell elastase in both diseases (Table I). Further, stool IL-1β correlated significantly with the endoscopic grade of UC ($P < 0.05$) (data not shown). Statistical association was also observed between IL-4 and IL-10 in the stool of CD patients and between IL-1β and IL-1ra in both UC and CD patients (Table I).

Stool cytokine concentrations in the active and ensuing inactive phase were compared in 7 patients with UC and 5 with CD. As shown in Fig. 2, IL-1β, IL-1ra, and TNFα decreased significantly after clinical resolution. Interestingly, IL-4, IL-10, and the ratio of IL-1ra to IL-1β were significantly increased after the resolution of disease exacerbation.

Stool cytokine concentrations were analyzed in relation to the disease location and medical treatment. Concentrations of all five cytokines were independent of the disease location in both forms of IBD (Table II). In addition, no significant differences in cytokine concentration were evident between active disease patients with steroid therapy and those without, except for IL-4 in UC (Table III).

**DISCUSSION**

There are many reports on cytokine profiles in the intestine of patients with IBD (5–8, 21–29). In the present investigation,
we focused on the appearance of cytokines in the stool of these patients.

We found increases in both IL-1 and its natural antagonist IL-1ra (30) in the stools of patients with active IBD and low levels in inactive disease and controls. This observation is similar to previous findings of increased tissue levels of IL-1 and IL-1ra in patients with IBD when using either whole biopsy specimens (24) or isolated lamina propria mononuclear cells (25). Moreover, we found a significant correlation of both IL-1 and IL-1ra with various clinical indicators of disease activities or severities. Although the design of this study did not enable further comparison of these two cytokines with the degree of disease activity, the present results suggest that stool IL-1 and IL-1ra are simple and relevant markers of IBD activity.

There is a general trend for the ratio of IL-1ra to IL-1 to be decreased in whole biopsy specimens (24) or cell extracts (25) from UC and CD patients when compared with controls. A similar trend was found in stool samples reported here. These observations suggest that inflammation could result from abnormal proportions of IL-1 and IL-1ra secondary to a relative excess of the former or insufficiency of the latter.

TNFz is a key inflammatory cytokine that shares several properties with IL-1 (31). Previous study in a pediatric population showed an increased TNFz concentration in the stools of patients with active IBD. Somewhat surprisingly, we could not find statistically significant differences in TNFz levels between the active and inactive CD groups. Tissue levels of TNFz have been measured in several studies with conflicting results (5, 6, 26). It is intriguing that TNFz is not consistently increased in IBD patients in a manner similar to that of the other pro-inflammatory cytokines, IL-1, IL-6, and IL-8. Differences in patient selection, tissue sampling, and diverse methods may in part explain these conflicting observations.

In contrast to other cytokines, the opposite situation arises for IL-4 (32, 33) and IL-10 (34, 35), both of which have the capacity to inhibit pro-inflammatory cytokines while stimulating IL-1ra. In a paired analysis of the same individual, stool concentrations of IL-4 and IL-10 have been shown to increase after the clinical resolution of disease exacerbation. Also, there was a significant correlation between stool concentrations of IL-4 and IL-10, especially in CD. The exact meaning of the delay in the increase in these two anti-inflammatory cytokines is unclear. However, it is conceivable that IL-4 and IL-10 may contribute to systemic down-regulation of the acute inflammatory process. Clinically, serial measurements in the same individual may help determine disease prognosis and confirm treatment efficacy. At present, conflicting results have been observed in tissue levels of both IL-4 and IL-10 in relation to disease activities (8, 27–29). Ideally, both intestinal tissue and stool samples should be evaluated simultaneously in the same patient.

It is well established that currently available drugs, particularly corticosteroids, attenuate inflammation in the bowel by suppressing the production of cytokines such as IL-1 and TNFz (36, 37). However, we could not find any difference in cytokine levels between patients with and

<table>
<thead>
<tr>
<th>Disease location</th>
<th>No. of patients</th>
<th>IL-1 β (log pg/g)</th>
<th>IL-1ra (log pg/g)</th>
<th>TNFα (log pg/g)</th>
<th>IL-4 (log pg/g)</th>
<th>IL-10 (log pg/g)</th>
</tr>
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<tr>
<td>Ulcerative colitis</td>
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<td></td>
<td></td>
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<tr>
<td>Proctitis</td>
<td>3</td>
<td>4.76 ± 0.33</td>
<td>5.04 ± 0.44</td>
<td>0.95 ± 0.38</td>
<td>2.06 ± 0.32</td>
<td>2.39 ± 0.24</td>
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<tr>
<td>Left-sided colitis</td>
<td>5</td>
<td>4.56 ± 0.27</td>
<td>5.26 ± 0.17</td>
<td>1.24 ± 0.36</td>
<td>2.15 ± 0.23</td>
<td>2.46 ± 0.36</td>
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<tr>
<td>Pancolitis</td>
<td>11</td>
<td>4.64 ± 0.42</td>
<td>5.14 ± 0.27</td>
<td>1.94 ± 0.18</td>
<td>2.56 ± 0.16</td>
<td>2.51 ± 0.27</td>
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<tr>
<td>Ileitis</td>
<td>7</td>
<td>2.89 ± 0.34</td>
<td>4.30 ± 0.36</td>
<td>0.92 ± 0.19</td>
<td>2.17 ± 0.27</td>
<td>2.44 ± 0.15</td>
</tr>
<tr>
<td>Colitis</td>
<td>3</td>
<td>5.08 ± 0.32</td>
<td>5.03 ± 0.17</td>
<td>1.30 ± 0.33</td>
<td>2.73 ± 0.69</td>
<td>3.14 ± 0.55</td>
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<tr>
<td>Ileocolitis</td>
<td>8</td>
<td>3.61 ± 0.43</td>
<td>4.26 ± 0.24</td>
<td>1.01 ± 0.25</td>
<td>2.04 ± 0.14</td>
<td>2.21 ± 0.15</td>
</tr>
</tbody>
</table>

IL = interleukin; TNF = tumor necrosis factor; IL-1ra = IL-1 receptor antagonist. Cytokine levels were compared using Bonferroni’s test with analysis of variance with logarithmically transformed data. The data represent mean ± standard error of the mean.

<table>
<thead>
<tr>
<th>Medication</th>
<th>No. of patients</th>
<th>IL-1 β (log pg/g)</th>
<th>IL-1ra (log pg/g)</th>
<th>TNFα (log pg/g)</th>
<th>IL-4 (log pg/g)</th>
<th>IL-10 (log pg/g)</th>
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<tbody>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>10</td>
<td>4.97 ± 0.36</td>
<td>5.39 ± 0.14</td>
<td>1.85 ± 0.17</td>
<td>2.44 ± 0.09</td>
<td>2.44 ± 0.24</td>
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<tr>
<td>Steroid*</td>
<td>4</td>
<td>4.82 ± 0.27</td>
<td>5.33 ± 0.30</td>
<td>1.27 ± 0.48</td>
<td>1.82 ± 0.25**</td>
<td>2.71 ± 0.41</td>
</tr>
<tr>
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<tr>
<td>None</td>
<td>9</td>
<td>3.01 ± 0.29</td>
<td>4.19 ± 0.30</td>
<td>0.87 ± 0.22</td>
<td>2.07 ± 0.22</td>
<td>2.36 ± 0.12</td>
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<tr>
<td>Steroid*</td>
<td>4</td>
<td>3.93 ± 0.59</td>
<td>4.58 ± 0.33</td>
<td>1.37 ± 0.03</td>
<td>2.63 ± 0.48</td>
<td>2.95 ± 0.41</td>
</tr>
</tbody>
</table>

IL = interleukin; TNF = tumor necrosis factor; IL-1ra = IL-1 receptor antagonist. Cytokine levels were compared using Student’s t test with logarithmically transformed data. The data represent mean ± standard error of the mean.

* Patients treated with steroid alone and both steroid and sulphasalazine.

** P < 0.05 versus ulcerative colitis patients without specific medication.
without steroid therapy, except for IL-4 in UC. Because of the small size of each subgroup, no final conclusion could be extrapolated with regard to the influence of steroids on stool cytokine levels. Further studies in a larger population are needed.

The question arose as to how cytokines appear in the stool. Cytokine that is produced locally in the diseased bowel may leak into the bowel lumen. Another possibility is that IBD-affected epithelial cells and immune-system cells, both capable of producing cytokines (25, 38), may be extruded into the bowel lumen and appear in the stool. The explanation that the stool cytokine level is mainly responsible for the leakage from the blood vessel within the gut wall is excluded by previous data showing 100-fold higher levels of various cytokines in stool than in plasma of patients with acute shigellosis (16).

In conclusion, measurement of cytokines in stool may provide a useful and noninvasive means of understanding of pathophysiology and clinical monitoring in IBD.

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