THE AMERICAN JOURNAL OF GASTROENTEROLOGY
Vol. 94, No. 12, 1999

ISSN 0002-9270/99/$20.00
Published by Elsevier Science Inc.
PII S0002-9270(99)00699-1

Fecal Eosinophil Granule-Derived Proteins Reflect Disease Activity in Inflammatory Bowel Disease

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OBJECTIVES: The aims of this study were: 1) to examine whether the fecal levels of eosinophil granule-derived proteins reflect disease activity in inflammatory bowel disease (IBD); and 2) to examine the extracellular release of these proteins from eosinophils and their stability in feces by an *in vitro* study.

METHODS: We investigated 42 patients with ulcerative colitis (UC), 37 patients with Crohn's disease (CD), and 29 control subjects. The stool samples were collected at 4°C over 48 h and were homogenized. The fecal levels of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) were measured by radioimmunoassay. Fecal Hb (Hb), α1-antitrypsin (AT), and lactoferrin (Lf) were also measured by ELISA.

RESULTS: Fecal ECP and EPX concentrations were significantly increased in both active UC and active CD compared to inactive UC and inactive CD, respectively. Fecal EPX concentration correlated with the fecal Hb, AT, and Lf concentrations more closely than fecal ECP concentration. Even in the inactive stage, CD patients who relapsed within the following 3 months showed higher fecal ECP and EPX concentrations compared to the patients who did not. EPX was released extracellularly more efficiently than ECP (18.6% *vs* 6.3%, after incubation for 15 min at 25°C). EPX was more stable in the feces than ECP.

CONCLUSIONS: The measurement of eosinophil granule-derived proteins in feces is useful for evaluating disease activity and predicting relapse in patients with IBD. EPX may be more suitable than ECP as a fecal eosinophil marker. (Am J Gastroenterol 1999;94:3513–3520. © 1999 by Am. Coll. of Gastroenterology)

INTRODUCTION

Eosinophils are involved in a broad range of diseases such as allergic, inflammatory, and malignant disorders (1, 2). The specific granules of the eosinophils contain a number of highly cationic proteins such as eosinophil cationic protein (ECP), eosinophil protein X (EPX)/eosinophil-derived neurotoxin (EDN), major basic protein (MBP), and eosinophil peroxidase (EPO). These proteins have potent cytotoxic action and are released from the cells after activation and stimulation of the cells (3). Intestinal mucosa of the patients with inflammatory bowel disease (IBD) is characterized by epithelial cell damage and infiltration of various inflammatory cells. The inflammatory cells include neutrophils, lymphocytes, plasma cells, macrophages, and eosinophils. Neutrophils contain various proteins such as lactoferrin, PMN (PMN)-elastase, myeloperoxidase, and lysozyme in their granules. We previously reported that the fecal levels of these neutrophil-derived proteins increased in feces in patients with active IBD and reflect disease activity (4, 5). We then considered that the measurement of fecal eosinophil-derived proteins may provide information regarding eosinophil involvement in the pathological process of IBD. The aims of the present study were: 1) to examine whether the fecal levels of eosinophil granule-derived proteins (ECP and EPX) reflect disease activity and predict relapse in patients with IBD; 2) to compare fecal eosinophil granule-derived proteins and other fecal markers of disease activity; and 3) to examine the extracellular release of eosinophil granule-derived proteins from eosinophils and their stability in feces by an *in vitro* study.

MATERIALS AND METHODS

Extracellular Release of ECP and EPX by Eosinophils In Vitro

A quantity of 500 μl of heparinized blood samples from four healthy subjects were incubated at 25°C for 15 min. Plasma was obtained by centrifugation at 2000 *g* for 5 min. The concentration of ECP and EPX in plasma was measured by a radioimmunoassay (RIA) kit (Pharmacia and Upjohn, Kalamazoo, MI). To determine the total amount of ECP and EPX in whole blood, Triton X-100 (1% final concentration) was added to the heparinized blood samples, and the concentrations of ECP and EPX were measured. The percentage of ECP and EPX released extracellularly was obtained by the following equation: Percent extracellular release (%) = (a/b) $\times$ 100, where a is the concentration (ng/ml) in plasma after incubation and b is the concentration (ng/ml) in whole blood treated with Triton X-100.

Subjects

A total of 42 patients with UC (age 34.5 ± 15.5 yr [mean ± SD]; eight with proctitis, 11 with left-sided colitis, 23 with pancolitis) and 37 patients with CD (age 29.1 ± 13.0 yr, 13 with the small intestine type, 18 with the small and large intestine type, six with the large intestine type) were evaluated. Crohn's colitis and UC were differentiated endoscopically and histologically. UC was defined as being in the active phase if the patients showed clinical symptoms (rectal bleeding, diarrhea) and/or an inflamed colonic mucosa (Baron's grade 2 or 3) at colonoscopy (6). Disease activity in CD was assessed according to the Crohn's disease activity index (CDAI), in which a score of >150 was considered to represent active disease (7). Regarding medication, sulfasalazine or 5-aminosalicylate was administered in 41 of 51 active UC samples, in 33 of 38 inactive UC samples, in 38 of 50 active CD samples, and in 34 of 49 inactive CD samples. Prednisolone was administered in 19 of 51 active UC samples, in 19 of 38 inactive UC samples, in 14 of 50 active CD samples, and in 15 of 49 inactive CD samples. The control group consisted of 29 subjects (age 40.6 ± 21.5 yr) with no endoscopic abnormality in the upper or lower digestive tract.

Informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

Method of Stool Collection and Measurement of Fecal ECP, EPX, Hb (Hb), α1-Antitrypsin (α1-AT), and Lactoferrin (Lf)

Patients were instructed to defecate directly into a polystyrene container (diameter 15 cm, depth 12 cm). The stool samples, stored at 4°C over 48–72 h, were homogenized with a small amount of water, and then stored at −80°C until the time of measurement. The fecal levels of ECP and EPX were measured by an RIA kit (Pharmacia and Upjohn). An RIA kit for ECP was a kind gift from Pharmacia and Upjohn. The optimal sample dilution for assay was first examined. Samples diluted at 1:40 or more with PBS showed good linearity of the assay system, whereas samples diluted at 1:20 with PBS showed a higher level than theoretically expected. This was considered to be due to possible interference present in the stool. Therefore, the samples were diluted at 1:40 or more with PBS. Coefficient of variations in intraday assay and interday assay for ECP and EPX were <15%. Fecal Hb, α1-AT and Lf were measured by ELISA as described previously (5, 8). Fecal Hb and α1-AT are useful markers of disease activity in UC and CD, respectively (5). Fecal Lf is useful particularly for evaluating the presence of minimal intestinal inflammation (8).

Stability of ECP and EPX in Feces

To examine the stability of these proteins in the feces, homogenized stool samples were stored at 4°C, 25°C, and 37°C for 0, 12, 24, and 48 h before freezing and subsequent analysis.

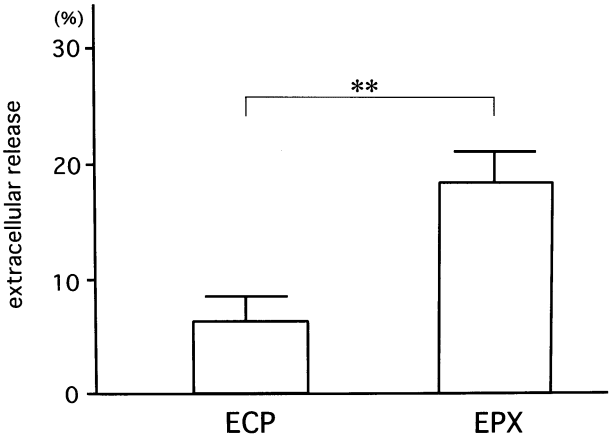

Figure 1. Extracellular release of ECP and EPX by eosinophils *in vitro*. Student's *t* test was used for statistical analysis: ** $p < 0.01$.

Contribution of Fecal Concentrations of ECP and EPX in the Inactive Phase of the Disease in Predicting Subsequent Relapse

Among the patients described in the Subjects section, 30 patients with UC (age 33.4 ± 15.0 yr [mean ± SD]; six with proctitis, nine with left-sided colitis, and 15 with pancolitis) and 35 patients with CD (age 29.1 ± 13.5 yr, 13 with the small intestine type, 17 with the small and large intestine type, and five with the large intestine type) were used as subjects to examine the contribution of fecal concentrations of ECP and EPX in predicting subsequent relapse. At the time of stool collection, all these patients had been at an inactive stage for >2 months. The patients were divided into "relapse patients" and "nonrelapse patients." There were no significant intergroup differences in the distributions of age and type of disease. Relapse was considered to occur when the disease became active. The definitions of active disease were mentioned above. A patient who relapsed within the 3 months after collecting stool samples was defined to be a "relapse patient." A patient who did not relapse within the 3 months after collecting stool was defined as a "nonrelapse patient." Fecal concentrations of ECP, EPX, Hb, α1-AT, and Lf were compared between "relapse patients" and "nonrelapse patients."

Statistical Analysis

Values were expressed as means ± SE. Student's *t* test was used for statistical analyses. Linear regression analysis was used for correlation analysis. All *p* values were two-tailed; values of $p < 0.05$ were considered statistically significant.

RESULTS

Extracellular Release of ECP and EPX by Eosinophils In Vitro

As shown in Figure 1, the extracellular release of ECP and EPX were 6.3 ± 2.0% and 18.6 ± 2.4%, respectively. The extracellular release of EPX was more efficient than that of

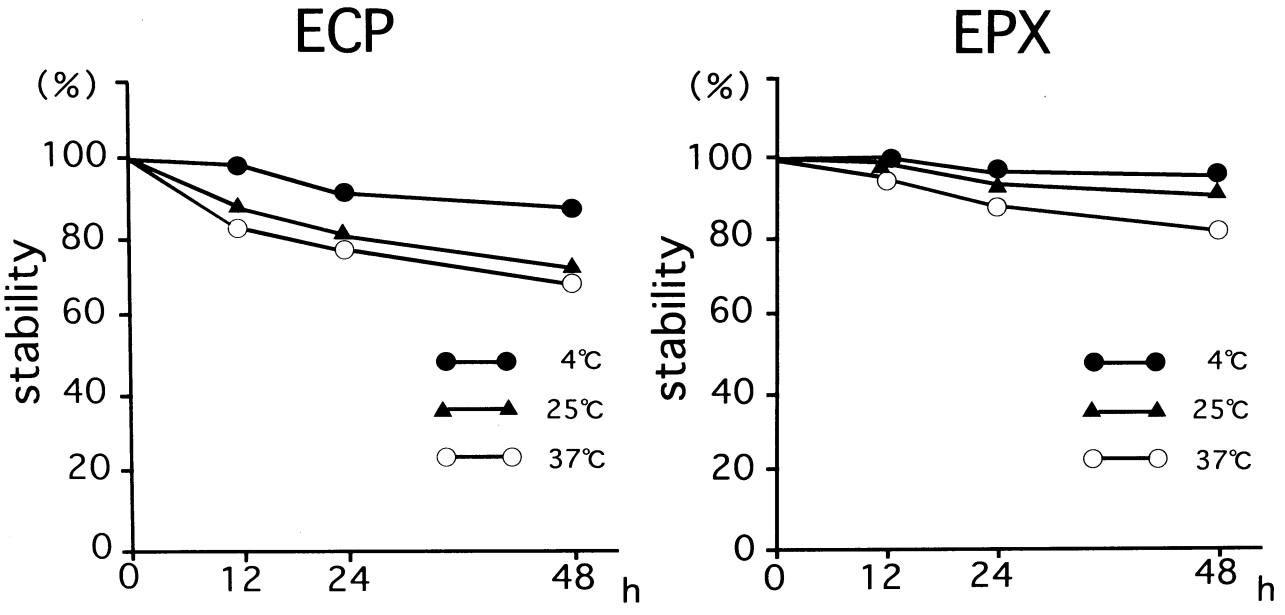

Figure 2. Stability of fecal ECP and EPX in feces: filled circles, at 4°C; &filled triangles, at 25°C; open circles, at 37°C.

ECP. After treatment with Triton X-100, the mean concentrations of ECP and EPX in whole blood were 138 and 171 μg/ml, respectively.

Stability of Fecal ECP and EPX

The data are shown in Figure 2. The concentration of ECP and EPX was expressed as a percentage of the original concentration at 0 h. EPX was more stable in the feces than was ECP (92.2% *vs* 73.0% at 25°C for 48 h).

Levels of Fecal ECP and EPX in Patients With UC and CD

Fecal concentrations of ECP and EPX in patients with UC and CD were demonstrated in Figures 3 and 4. In patients

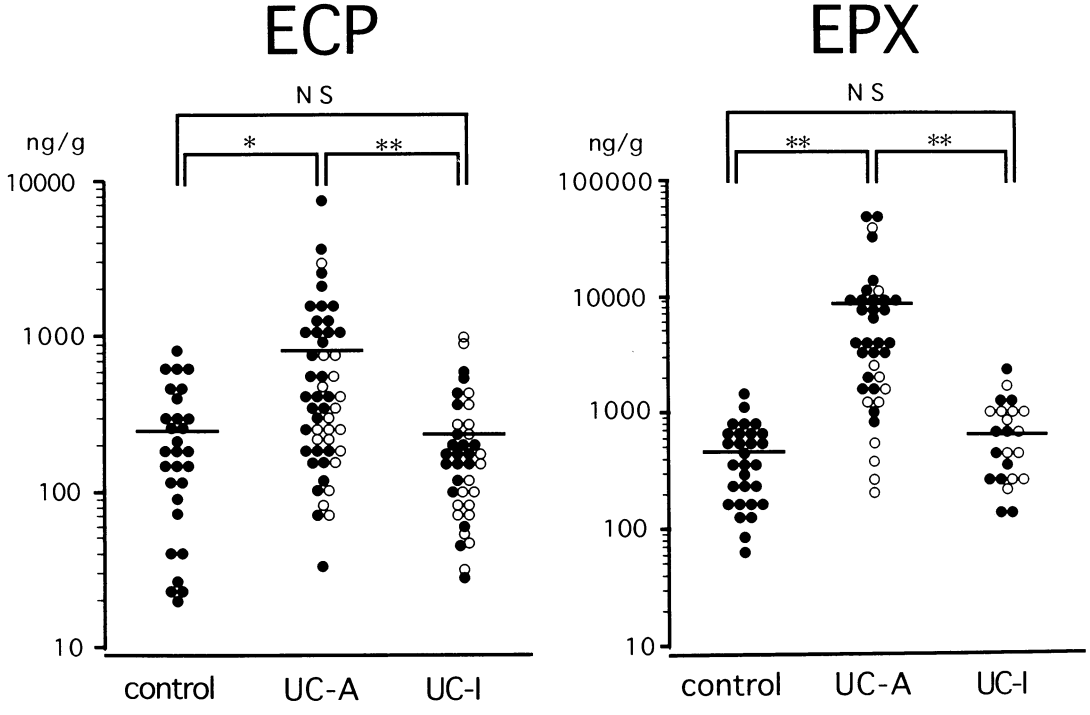

Figure 3. Fecal concentrations of ECP and EPX in patients with UC. UC-A, ulcerative colitis (active phase); UC-I, ulcerative colitis (inactive phase). Patients who did not receive corticosteroids are indicated by filled circles, and patients who received corticosteroids are indicated by open circles. When the patients who received corticosteroids were excluded from the subjects, the same differences were found. Student's *t* test was used for statistical analyses: * $p < 0.05$; ** $p < 0.01$. NS = not significant.

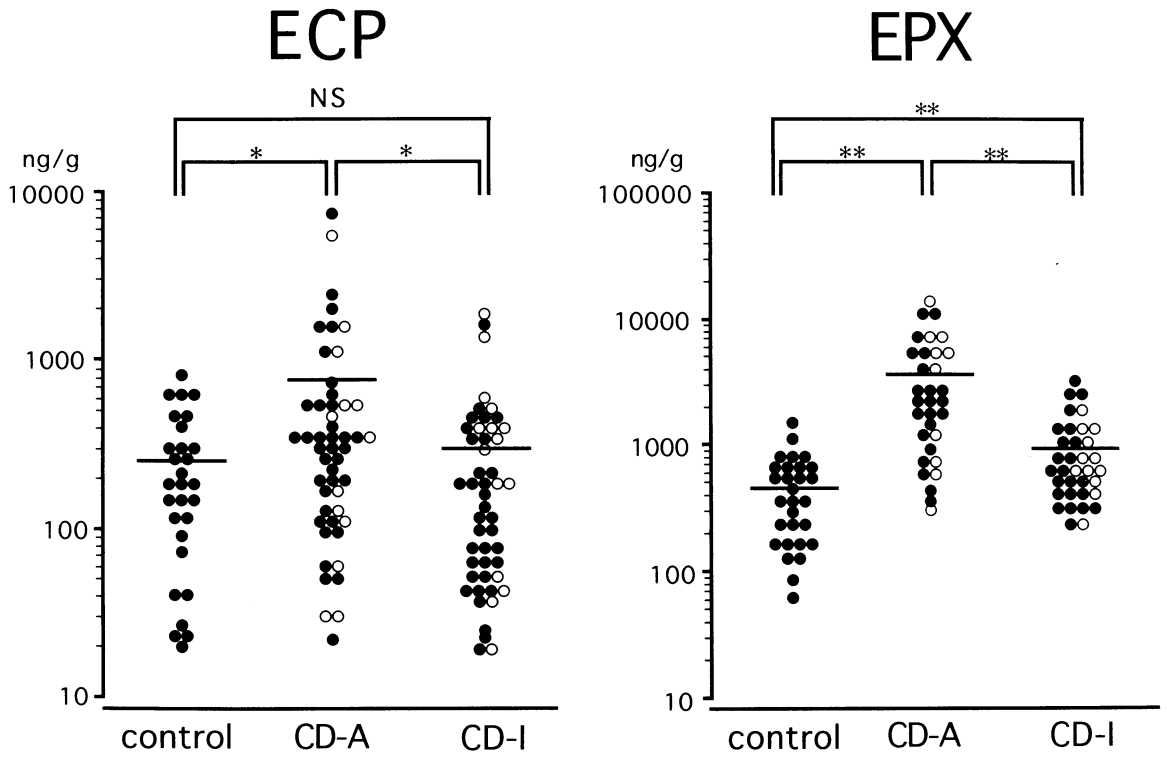

Figure 4. Fecal concentrations of ECP and EPX in patients with CD. CD-A; Crohn's disease (active phase); CD-I, Crohn's disease (inactive phase). Patients who did not receive corticosteroids are indicated by filled circles, and patients who received corticosteroids are indicated by open circles. When the patients who received corticosteroids were excluded from the subjects, the same differences were found. Student's *t* test was used for statistical analyses: * $p < 0.05$; ** $p < 0.01$. NS = not significant.

with active UC, inactive UC, active CD, inactive CD and in the control subjects, fecal ECP concentrations were 822.1 ± 169.3, 239.3 ± 34.9, 785.4 ± 194.9, 301.9 ± 52.4, and 248.4 ± 39.3, respectively. Respective fecal EPX concentrations were 8576.3 ± 2043.5, 654.4 ± 118.4, 3522.9 ± 604.7, 910.2 ± 123.7, and 455.0 ± 61.4. In both UC and CD, there were significant differences between the active and inactive phases, and between the active phases and the control. When patients who received corticosteroids were excluded from the subjects, the same differences were found. Fecal EPX showed more evident difference between the disease group and the control than fecal ECP. Patients with active UC were further divided into two groups: patients with massive bleeding (daily fecal Hb excretion > 0.5 g/day) and patients without massive bleeding. Patients with active CD were divided into two groups according to their CDAI score. In patients with active UC (massive bleeding, n = 10), active UC (no massive bleeding, n = 41), active CD (CDAI > 200, n = 12) and active CD (150 < CDAI ≤ 200, n = 37), the fecal ECP concentrations were 945.4 ± 236.8, 792.0 ± 203.4, 884.0 ± 578.1, and 682.2 ± 176.4, respectively. The respective fecal EPX concentrations were 22308.7 ± 6735.0, 4788.0 ± 1162.5, 4098.4 ± 1191.5, and 3240.1 ± 742.5. There were significant correlations between the fecal levels of eosinophil granule-derived proteins and daily fecal Hb excretion in UC ($r = 0.400$, $p < 0.001$ for ECP, $r = 0.745$, $p < 0.001$ for EPX). Between the active UC with massive bleeding and those without massive bleeding, there was a significant intergroup difference in the fecal EPX concentration, but not in the fecal ECP concentration. In CD, there was a significant correlation between the fecal EPX concentration and CDAI ($r = 0.505$, $p < 0.001$), but not between the fecal ECP concentration and CDAI ($r = 0.202$, $p = 0.053$). Between active CD (CDAI > 200) and active CD (150 < CDAI ≤ 200), however, there was no significant intergroup difference in fecal ECP or EPX concentrations.

Relationship Between Fecal ECP and EPX in Patients With UC and CD

The values are shown in Figure 5. There were significant correlations on the logarithmic scale between ECP and EPX concentrations in UC and CD. UC showed better correlation than CD. Most of the samples that showed a normal ECP concentration and a high EPX concentration were obtained from active patients (18 of 21 in UC, and 16 of 25 in CD).

Relationship Between Fecal Eosinophil Markers and Hb or α1-AT in Patients With UC and CD

We previously found that fecal Hb and α1-AT were useful markers of disease activity in UC and CD, respectively. We therefore examined the relationship between concentrations of the eosinophil markers (ECP, EPX) and the concentrations of Hb or α1-AT. As shown in Figure 6, there were

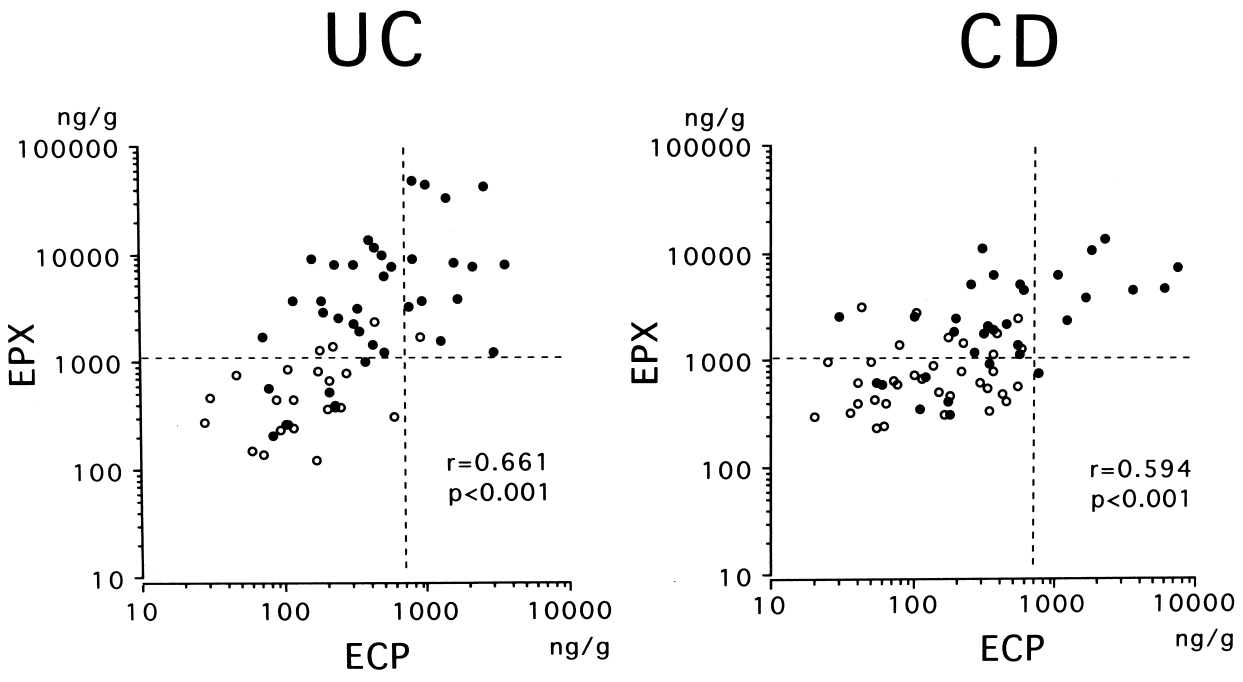

Figure 5. Relationship between fecal ECP and EPX in patients with UC and CD. Dotted lines, mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g for ECP, EPX, respectively). Open circles, inactive phase; filled circles, active phase.

significant correlations on the logarithmic scale between eosinophil markers and Hb or α1-AT in UC and CD. Fecal EPX concentrations correlated with the fecal Hb or AT concentrations more closely than did the fecal ECP concentrations. Good correlations were obtained in UC, but not in CD.

Relationship Between Fecal Eosinophil Markers and Lf in Patients With UC and CD

The values are shown in Figure 7. There were significant correlations on the logarithmic scale between fecal eosinophil markers and Lf in UC and CD. UC showed better correlation than CD. Fecal EPX concentrations correlated with the fecal Lf concentrations more closely than did the fecal ECP concentrations.

Predictive Value of Fecal Eosinophil Markers for Subsequent Relapse

As shown in Figure 8, in patients with CD, fecal ECP and EPX showed significant differences between "relapse patients" and "nonrelapse patients." In UC, there were no significant differences between "relapse patients" and "nonrelapse patients." However, UC patients with high levels of fecal eosinophil markers relapsed. In contrast to fecal eosinophil markers, fecal Hb, α1-AT, and Lf did not show significant differences between "relapse patients" and "nonrelapse patients" in either UC or CD (data not shown).

DISCUSSION

In patients with various intestinal diseases including IBD, increased eosinophil counts and enhanced eosinophil activation were found in the intestinal mucosa (9–11). Because there has been no appropriate method to assess the eosinophil activation of the intestinal mucosa, the role of eosinophils in the pathogenesis of IBD remains unclear. Routine histological observation of the intestinal mucosa misses degranulated eosinophils and is not sufficient for the assessment of eosinophil activation. Immunohistochemistry using antibody against eosinophil granular proteins is useful for assessing eosinophils of the intestinal mucosa (9). However, colonoscopy is required to obtain biopsies of the intestinal mucosa. In contrast, fecal tests are safe and can be performed repeatedly. To establish the measurement of fecal eosinophil markers is important from a clinical point of view. Eosinophils produce and release various inflammatory mediators. Among them, a number of highly cationic proteins present in the granules of the eosinophils are specific for eosinophils. In the present study, therefore, we focused on fecal eosinophil granule-derived proteins as a marker of eosinophil activation of intestinal mucosa.

There have been two reports concerning fecal eosinophil granule-derived proteins (13, 14). Comparing the two studies, Berstad *et al.* reported approximately 20-fold higher fecal levels of ECP in feces than did Bischoff *et al.* The present study demonstrated that the assay system had good linearity and a reasonable coefficient of variations. The fecal ECP levels of the present study were closer to that reported by Berstad *et al.* Both reports showed high fecal levels in patients with active IBD. However, they did not characterize inactive patients who had high fecal levels of eosinophil markers. The usefulness of fecal eosinophil markers for predicting relapse was not examined. In addition, we com-

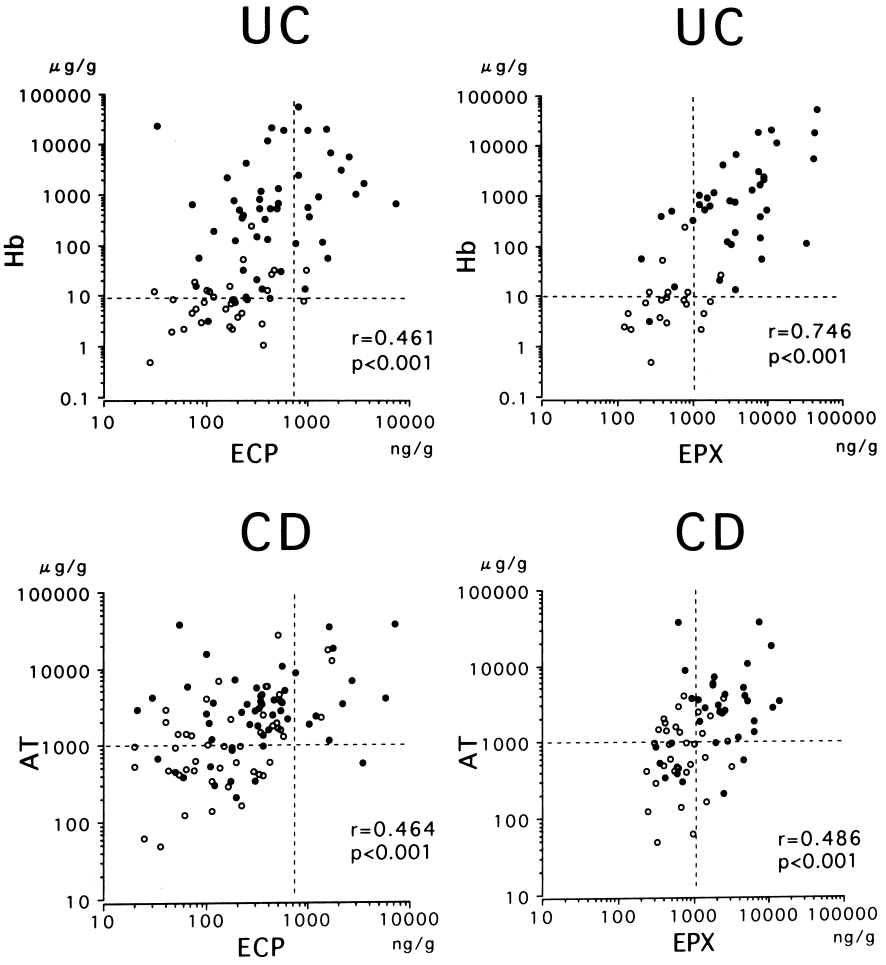

Figure 6. Relationship between fecal eosinophil markers and Hb or α1-AT in patients with UC and CD. Dotted lines, mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g, 9.7 μg/g, and 995.5 μg/g for ECP, EPX, Hb, and α1-AT, respectively). Open circles, inactive phase; filled circles, active phase.

pared ECP and EPX in terms of their stability in feces as well as their extracellular release.

It is well known that eosinophil granular proteins are released extracellularly when the cells are stimulated. To constitute a superior fecal marker of intestinal eosinophil activation, a protein should be released efficiently from the cells, as well as being stable in feces. From this point of view, EPX seemed to be a more suitable marker than ECP, because it was released from the cells more efficiently and was more stable in the feces.

Fecal Hb, α1-AT, and neutrophil-derived proteins are markers for disease activity in IBD (4, 5, 8). Fecal Hb is good for UC, whereas α1-AT is good for CD. Lf is a useful marker for evaluating the presence of minimal intestinal inflammation of UC and CD. Hb and Lf were elevated in almost all of the patients with active UC, indicating that bleeding and mucosal neutrophil infiltration are common features of all patients with UC. In the present study, however, fecal EPX and ECP were not elevated in all of the patients with active UC, indicating that activation of intestinal eosinophils is not a common feature of all patients with IBD. Fecal ECP and EPX levels reflect disease activity to some extent. However, fecal ECP and EPX levels may provide information on eosinophil activation of the intestinal mucosa, rather than on quantitative disease activity. Clinical features may be different between IBD patients who have activated intestinal eosinophils and IBD patients who do not. It would be interesting to note whether or not there are similarities in the clinical features of IBD patients who have activated intestinal eosinophils and patients with eosinophilic gastroenteritis. Furthermore, modified or optional medical treatment may be useful in the subgroup of IBD patients with strong activation of intestinal eosinophils. Drugs that inhibit the migration and activation of eosinophils may be useful as maintenance therapy to prevent recurrence in these patients (16, 17). Medical treatment may be a relevant factor affecting fecal ECP and EPX levels. In the present study, even if the patients who received corticosteroids are excluded from the subjects, fecal ECP and EPX levels were significantly increased in both active UC and active CD compared to inactive UC and inactive CD, respectively.

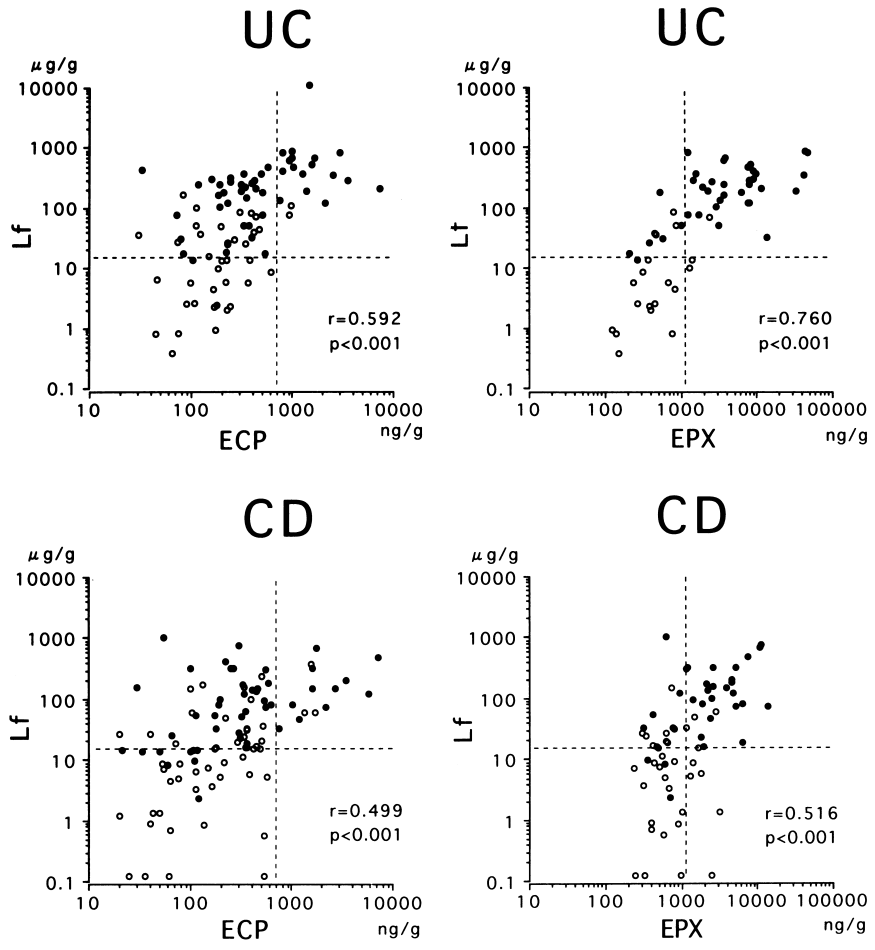

Figure 7. Relationship between fecal eosinophil markers and Lf in patients with UC and CD. Dotted lines, mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g, and 16.5 μg/g for ECP, EPX, and Lf, respectively). Open circles, inactive phase; filled circles, active phase.

UC and CD are relapsing and remitting diseases. Factors predisposing to recurrence are poorly understood. In the active phase of IBD, the participation of eosinophils in the pathophysiology is clear (17–20). It was reported that large numbers of eosinophils in the rectal mucosa during the active disease predict a benign course. However, it had been unknown whether eosinophils contribute to early mucosal damage in patients with IBD. Recently, Dubucquoi *et al.* showed that eosinophil infiltration was detected in early endoscopic recurrence cases after radical resection for CD (21). More recently, D'Haens *et al.* demonstrated that contact with intestinal fluids induced focal infiltration of mononuclear cells and eosinophils in the ileum of patients with CD (22). Our findings showed that patients with CD who relapsed clinically within the following 3 months showed higher fecal ECP and EPX concentrations even in the inactive phase. These findings indicate that eosinophil activation of the intestinal mucosa may trigger a flare-up of inflammation, which leads to clinical relapse. To address the accurate causal relation between eosinophil activation and relapse, the fecal levels of eosinophil markers should be examined serially and chronologically in patients with IBD.

It has been suggested that the results of immunological tests on extracts of feces do not represent the status of the gut humoral system (23). In the present study, there was substantial overlap of the fecal concentrations of EPX and ECP between the active and inactive phases. Analyses of whole gut lavage may be more accurate than fecal tests for estimating the total amount of mediators released into the gut lumen (14, 23), but the methods are complicated.

In conclusion, the measurement of eosinophil granule-derived proteins in feces is useful for evaluating the disease activity and predicting relapse in patients with IBD. EPX may be more suitable than ECP as a fecal eosinophil marker.

ACKNOWLEDGMENTS

The authors thank Drs. Hisashi Matsumoto, Kentaro Maemura, Seigou Tanaka, and Tsutomu Teranishi for their helpful discussions. This work was supported in part by a Grant-in aid 07670630 (to O.S.) for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan.

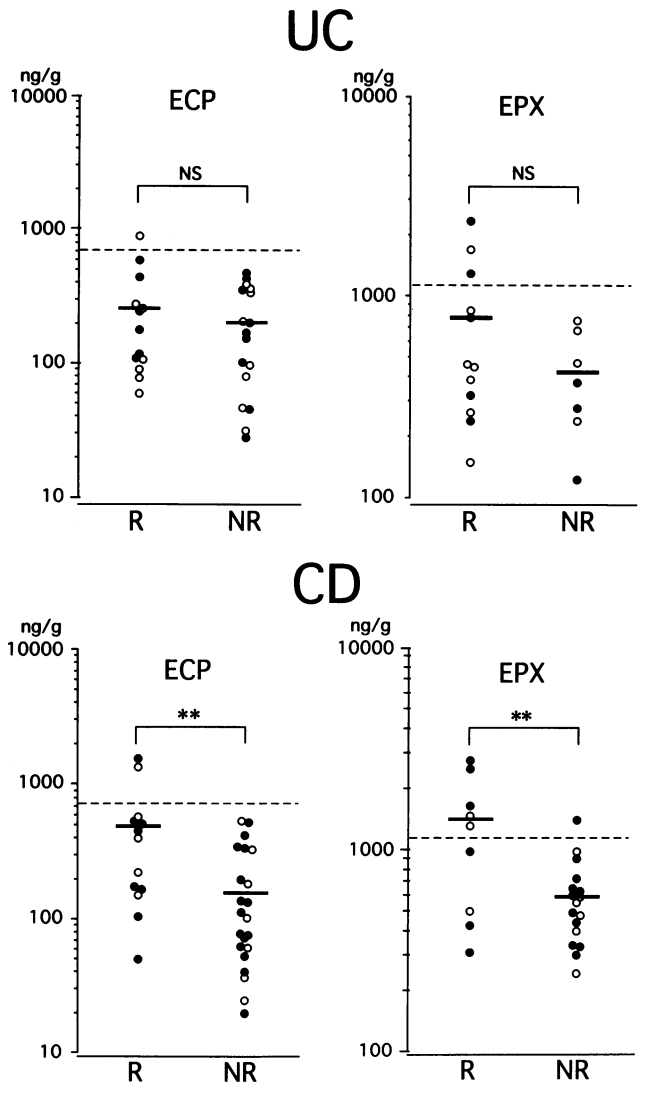

Figure 8. Fecal concentrations of ECP and EPX in inactive UC and CD: Comparison between relapse patients and nonrelapse patients. R = relapse within the following 3 months, NR = nonrelapse within the following 3 months. Dotted lines indicate mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g for ECP, EPX, respectively). Patients who did not receive corticosteroids are indicated by filled circles, and patients who received corticosteroids are indicated by open circles. When the patients who received corticosteroids were excluded from the subjects, the same differences were found. ** $p < 0.01$. NS = not significant.

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Received Oct. 23, 1998; accepted Apr. 19, 1999.

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