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A New Method for the Quantification of Neutrophil and Eosinophil Cationic Proteins in Feces: Establishment of Normal Levels and Clinical Application in Patients With Inflammatory Bowel Disease

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OBJECTIVES: The aims of this study were 1) to develop a valid method for the measurement of the eosinophil proteins eosinophil cationic protein (ECP) and eosinophil protein X (EPX) and neutrophil proteins myeloperoxidase and human neutrophil lipocalin (HNL) in feces and 2) to investigate their potential role as disease activity markers in inflammatory bowel disease (IBD).

METHODS: Feces samples were obtained from 44 apparently healthy individuals (HIs), 18 patients with IBD (11 with ulcerative colitis [UC] and seven with Crohn's disease [CD]), and three with collagen colitis. The granulocyte markers were measured using immunoassays in supernatants from processed feces.

RESULTS: ECP, myeloperoxidase, and, to a lesser degree, EPX and HNL were bound to the solid part of feces. However, feces homogenized in an extraction buffer containing the cationic detergent *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide allowed an efficient recovery of the proteins (*i.e.*, up to 100-fold increased levels compared to homogenization in saline). All four proteins were stable for at least 7 days at +6°C and at least 3 days at +22°C. The normal fecal geometric mean (95th percentile) levels of ECP, EPX, myeloperoxidase, and HNL were estimated to be, respectively, 1.69 μg/g (6.41), 0.57 μg/g (1.72), 3.54 μg/g (8.77), and 1.97 μg/g (4.91). Markedly increased feces levels of all markers ($p < 0.0002$), compared to HIs and CD patients, were observed in UC. However, the marker levels in CD patients were significantly increased relative to HIs ($p < 0.05$ to $p < 0.0002$). Increased levels of HNL and myeloperoxidase were also observed in the three collagen colitis patients. The discriminative capability between UC patients and HIs was somewhat superior for EPX and myeloperoxidase.

CONCLUSIONS: The method described here takes into account the molecular properties of the granule proteins and the heterogeneity in feces consistency, which is a prerequisite for a valid and reproducible measurement of cationic granule proteins. We suggest that EPX and myeloperoxidase, when applied in IBD, are the best eosinophil and neutrophil markers for studying GI inflammation. (Am J Gastroenterol 2002;97:1755–1762. © 2002 by Am. Coll. of Gastroenterology)

INTRODUCTION

Eosinophils and their granule proteins eosinophil cationic protein (ECP) (1) and eosinophil protein X (EPX) (2) are generally recognized as being involved in the host defense against invading parasites. They are markedly cationic proteins with cytotoxic capacities probably leading to tissue destruction as well as modulators of the immune response (3). A possible role of the eosinophil in several intestinal diseases has been suggested. Patients with celiac disease have been shown to have prominent infiltration of eosinophils in the lamina propria, and activation of eosinophils was suggested by the release of ECP in tissue and lumen of the intestine (4, 5). The eosinophil may also be a major actor in the pathogenesis of inflammatory bowel disease (IBD) because bowel biopsies from patients with IBD have demonstrated an infiltration of eosinophils in the lamina propria (6–10) and marked extracellular deposits of ECP (10). There is also an excess release of the eosinophil proteins ECP and EPX in luminal fluid and feces of patients with active ulcerative colitis (UC) or Crohn's disease (CD) (11–14).

The neutrophil granule protein human neutrophil lipocalin (HNL), which is stored in secondary granules (15), seems to be the most specific marker for the neutrophil (16). Myeloperoxidase, on the other hand, is stored in and released from the primary granules (17). Studies on the pathogenesis of IBD have also revealed an increased recruitment of neutrophils to the intestinal mucosa during active disease

(18) and release of neutrophil granule proteins such as myeloperoxidase and lysozyme in the mucosa (19) and lumen (4, 20).

Studying local inflammation such as in the GI tract involves invasive procedures like endoscopy and biopsy sampling. Simple noninvasive procedures are needed, and attempts have been made to quantify inflammation by measuring markers in faeces (11, 12).

The present study has focused on the validity of measurement of the eosinophil granule proteins ECP and EPX and the neutrophil proteins myeloperoxidase and HNL in feces. The method was applied in healthy individuals in whom normal levels were established and in patients with IBD.

MATERIALS AND METHODS

Healthy Controls and Patients

Feces samples were obtained from 44 apparently healthy individuals and 21 patients with IBD.

A questionnaire including health status was filled in by each apparently healthy individual. Individuals who were considered healthy were, within the scope of the questionnaire, not suffering from thyroid disease, heart and vascular disease, tumors, joint disease, diabetes, liver disease, lung disease, allergic disease, dermatitis or eczema, food allergy, IBD or other GI disease, frequent urinary infection, or other recent infection and not receiving anti-inflammatory treatment. The median age (range) of the healthy donors was 43.5 yr (18–73).

Patients included in the study were either hospitalized or on a regular clinic visit. The diagnoses of UC (n = 11) and CD (n = 7) were based on established criteria including a typical endoscopic/radiological appearance supported by histological findings (21). Ten of the UC patients had colitis, and one patient had isolated proctitis. The diagnosis of collagen colitis (CC; n = 3) was based on a history of chronic or intermittent watery diarrhea and typical histopathological findings, with cell infiltrates in the colonic epithelium and thickening of more than 10 μm in the subepithelial collagenous layer. The CC patients had normal endoscopic appearance of the colon.

Disease activity at the time of sample collection was assessed using the American Crohn's Disease Activity Index (CDAI) (22) for CD, the clinical and endoscopic scores according to Binder (23) for UC, and number of bowel movements per day for CC.

The clinical symptoms of the UC patients varied from normal to very active disease and were in most cases in agreement with the endoscopic findings. Accordingly, two patients had severe disease activity, seven patients had moderate activity, and two patients had mild activity. Eight patients were taking 5-aminosalicylic acid (5-ASA)–containing drugs, two patients were in addition to 5-ASA taking *p.o.* steroids or azathioprine, and one patient had no medication at the time of investigation.

Four of the CD patients were considered to have active disease (CDAI > 150), whereas three patients were considered to be in remission. Three patients were taking 5-ASA and steroids/azathioprine, one patient was taking steroids/azathioprine, one patient had recently, in addition to 5-ASA and steroids/azathioprine, been taking anti–tumor necrosis factor (infliximab), and one patient was on no medication at the time of investigation.

One of the patients with CC was considered to have severe disease, whereas the others had mild to moderately active disease. One patient was taking a nonsteroidal anti-inflammatory drug and two patients had no medication.

Preparation of Stool Sample Extracts

Stool samples were collected randomly and either immediately frozen at −27°C or stored in the refrigerator for a maximum of 12 h before freezing. Feces samples were thawed overnight in the refrigerator or at room temperature using a fan. Approximately 0.1–1 g of feces was weighted and diluted five times by adding 4 volumes (vol/wt) of an extraction buffer consisting of phosphate-buffered saline, pH 7.4, supplemented with 10-mmol/L ethylenediaminetetraacetic acid, 0.2% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB), 20% glycerol, 0.05% Tween20, and 1% bovine serum albumin. The mixture was homogenized using a Polytron PT1200 CL mixer (Kinematica, Lucerne, Switzerland) for 5–90 s until a homogenous solution was obtained. An aliquot of 0.5 ml of the homogenate was thereafter further diluted 20 times in extraction buffer. After incubation at 6°C for 30 min and mixing, the homogenate was centrifuged at 20,800 × g for 30 min at 5°C. The supernatant without particles was thereafter transferred to three tubes and frozen at −27°C for later analysis.

Two separate tubes containing 1500 μl of the 1:5 diluted homogenate were weighed and centrifuged at 20,800 × g for 30 min at 5°C. By weighing the pellet obtained after discarding the supernatant, a measure of semidry weight was obtained.

Stool samples were also, for comparison with the extraction buffer, mixed in 0.15-mol/L NaCl.

Assessment of ECP, EPX, Myeloperoxidase, and HNL

ECP was determined using the Pharmacia CAP System (ECP CAP FEIA, Pharmacia Diagnostics, Uppsala, Sweden), and EPX and myeloperoxidase were determined using commercial radioimmunoassays (Pharmacia Diagnostics) according to the manufacturer's instructions. The concentrations of HNL (μg/L) in the supernatant were determined using a prototype immunofluorometric assay utilizing the Pharmacia CAP System as previously described (24). All measurements were performed in duplicates of 50 μl of the supernatants and without knowledge of the diagnosis and clinical status of the patients.

Stability of ECP, EPX, Myeloperoxidase, and HNL in Feces

Portions of five unprocessed feces samples were kept frozen after the day of collection at −27°C or incubated at +6°C,

+22°C, and +34°C for 1, 2, 3, and 7 days. Supernatants obtained after processing were kept frozen or incubated at +6°C for 1, 3, and 7 days. Separate portions of feces and supernatants were also repeatedly frozen and thawed throughout the study.

Recovery of ECP, EPX, Myeloperoxidase, and HNL in Feces

Purified ECP, EPX, myeloperoxidase, and HNL for recovery experiments were obtained as previously described (2, 15, 25, 26). Three feces samples containing 1.30, 24.4, and 1.73 μg of ECP/g of feces; 1.45, 9.37, and 0.67 μg of EPX/g; 15.6, 202, and 1.65 μg of myeloperoxidase/g; and 5.73, 51.7 and <4.04 μg of HNL/g were obtained from two patients with IBD and one healthy individual, respectively. Approximately 3.5 g of each sample was mixed with 4 parts of 0.15-mol/L NaCl and suspended in aliquots of 1 ml. One hundred microliters of buffer alone or purified ECP, EPX, myeloperoxidase, and HNL in 0.15-mol/L NaCl were thereafter added at a final concentration of 4.61, 3.51, 14.0, and 11.6 mg/L to the aliquots and in duplicates. The same amounts of purified proteins were added to 1 ml of 0.15-mol/L NaCl. Each aliquot was thereafter diluted 20 times with the extraction buffer, and the feces samples were centrifuged at 40,000 × g for 60 min. Supernatants were kept frozen until analyzed.

Extractability and Linearity of the Measurement of the Inflammatory Markers in Feces

Stool samples from five healthy individuals and from eight patients with IBD were diluted either with 4 parts of 0.15-mol/L NaCl or 99 parts of extraction buffer to compare the extractability of these buffers. Samples were vigorously mixed and incubated at 6°C for 1 h and thereafter centrifuged at 20,800 × g for 30 min at 5°C. Supernatants extracted with 0.15-mol/L NaCl were further diluted with extraction buffer before analysis.

Time dependency of the extraction was examined by mixing six feces samples with 4 parts of 0.15-mol/L NaCl and then incubating aliquots of the samples with 19 parts of extraction buffer at 4°C for 30 min, 1 h, 2 h, 4 h, and 7 h, respectively. The samples were thereafter centrifuged at 20,800 × g for 30 min at 5°C. Supernatants were kept frozen at −27°C until analysis.

Statistical Methods Used for Evaluation

The Statistica software package (StatSoft, Tulsa, OK) was used for all statistical analyses. Comparison of the inflammation marker levels between different storage conditions was performed using analysis of variance (ANOVA) on ln-transformed data followed by a least significant difference *post hoc* test, and comparison between study groups was performed using ANOVA on ln-transformed data followed by a *post hoc* test (Tukey honestly significant difference test for unequal N). Spearman rank correlation (R_s) was used to express relationship between disease activity scores and markers. Grouped data were expressed as geometric means and percentiles or medians and ranges. Grubb's test at the 1% significant level was used for detecting outliers.

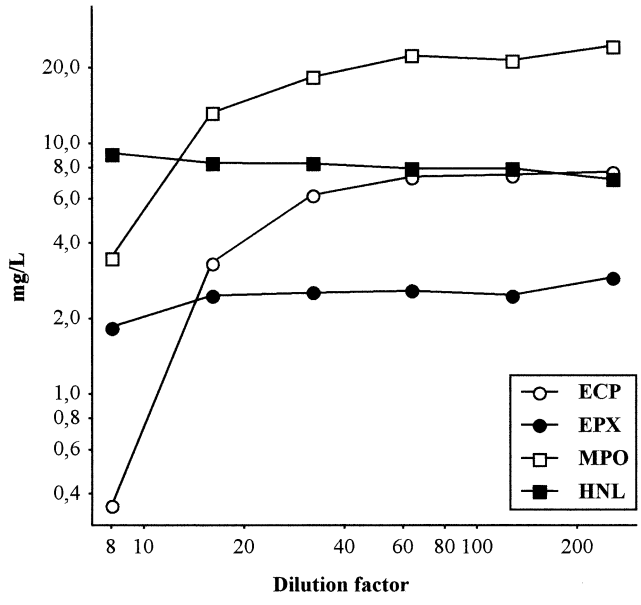

Figure 1. Recovery of ECP, EPX, myeloperoxidase (MPO), and HNL in supernatants of feces after extraction. Feces from one healthy individual were dispersed in various volumes of extraction buffer and incubated for 3 h at +6°C.

RESULTS

Extraction Efficacy and Recovery of ECP, EPX, Myeloperoxidase, and HNL in Feces

To assure an effective recovery of eosinophil and neutrophil granule markers from feces several extraction media, including buffer solutions with nonionic or cationic detergents and solutions without detergents, were examined (not shown). A clear relationship between extraction volume and extraction efficacy was observed, as shown in Figure 1, using the most effective extraction buffer (see Materials and Methods). For the two most cationic proteins, ECP and myeloperoxidase, a final dilution of at least 100 times was required to obtain an optimal extraction recovery.

Six feces samples that were diluted 1:100, dispersed, and incubated in extraction buffer for 0.5–7 h showed no time-dependent variation of the granulocyte marker levels (results not shown).

The levels of granulocyte markers after dispersion of feces in 0.15-mol/L NaCl and thereafter in the extraction buffer are shown in Figure 2. The second extraction of feces with extraction buffer yielded overall a substantial additional amount of ECP (geometric means = 0.051 *vs* 2.43 mg/L) and myeloperoxidase (geometric means = 0.65 *vs* 8.11 mg/L). However, for EPX (geometric means = 0.56 *vs* 0.85 mg/L) and HNL (geometric means = 12.68 *vs* 3.44

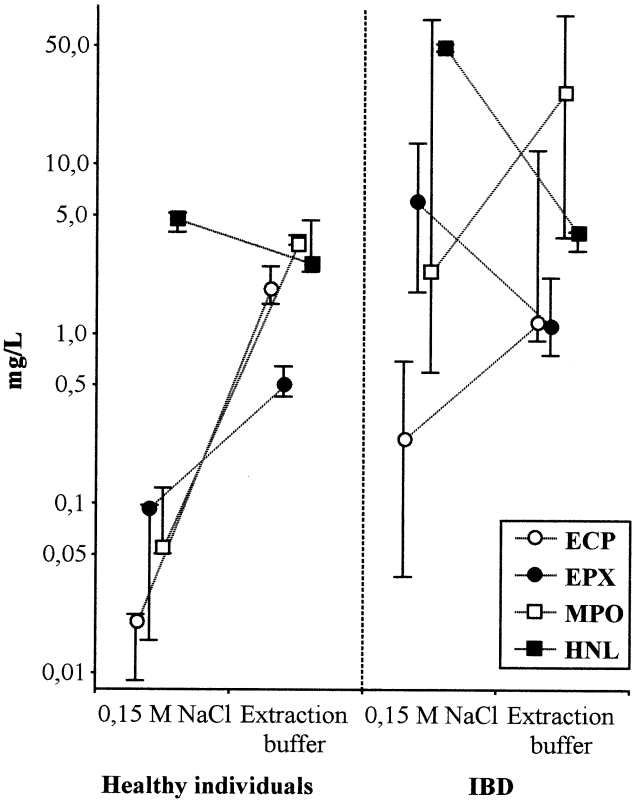

Figure 2. Effect of dispersion of feces in 0.15-mol/L NaCl and extraction buffer on the recovery of ECP, EPX, myeloperoxidase (MPO), and HNL in supernatants. Feces samples from healthy individuals (n = 9) and IBD patients (n = 10) were processed in 4 volumes (vol/wt) of 0.15-mol/L NaCl. After centrifugation the pellets were processed in 99 volumes of extraction buffer. Data are presented as medians and 25–75% ranges.

mg/L) the yields after extraction were more heterogeneous between the study groups (Fig. 2).

The median (minimum, maximum) recoveries after addition of purified proteins were 88.9% of expected (85.7, 106) for ECP, 99.4% (91.2, 112) for EPX, 107% (89.8, 110) for myeloperoxidase, and 111% (>83, 119) for HNL. Much lower recoveries were observed with ECP and myeloperoxidase when extraction buffer was substituted with 0.15-mol/L NaCl during dilution or extraction of feces.

The observation that a substantial amount of ECP and myeloperoxidase was bound to the solid part of feces prompted us to adjust the marker levels for water content. This was made feasible by weighing both the unprocessed feces sample and the semidry pellet obtained after centrifugation of the diluted sample and then adjusting the marker levels according to the ratio between the two weights. In feces from healthy individuals the granulocyte marker levels adjusted for semidry weight were almost identical to the measured levels (geometric mean ratio = 0.97). In patient samples, however, because of the higher water content, the adjustment resulted in a substantial increase in granulocyte marker levels (geometric mean ratio = 3.7).

Stability and Repeatability of ECP, EPX, Myeloperoxidase, and HNL Markers

The stability of the granulocyte markers was examined on both feces samples and feces supernatants. The results showed, with respect to marker levels, that all markers are stable in feces for 7 days in a refrigerator (+6°C). At room temperature (+22°C) all markers were stable for 7 days except ECP, which remained stable for 3 days. More pronounced effects could be seen after storage at +34°C, ECP, myeloperoxidase, and HNL being stable for, respectively, 2, 1, and <1 days, whereas EPX was still stable after 7 days of storage (Fig. 3).

In supernatants from processed feces, the markers showed no tendency to instability after storage for 7 days in a refrigerator (+6°C) because the levels of all four markers at day 7 diverged less than 10% from the prestudy levels. Moreover, three cycles of freezing at −27°C and thawing of feces or supernatants did not affect any of the studied markers.

Sampling of feces from one healthy individual five times during 8 months revealed intersampling coefficient variations (CVs) of 20% for ECP, 12% for EPX, 31% for myeloperoxidase, and 27% for HNL. Moreover, measurements of ECP, EPX, myeloperoxidase, and HNL in six portions of 0.1 g each taken from one stool sample showed CVs of 9.73%, 11.04%, 7.01%, and 9.69%, respectively. No differences in CVs were observed when 1 g was taken instead of 0.1 g.

Feces Levels of Granulocyte Markers in Apparently Healthy Individuals and Patients With IBD

The normal levels of granulocyte markers expressed as geometric means (95th percentile) were 1.69 μg/g (6.41) for ECP, 0.57 μg/g (1.72) for EPX, 3.54 μg/g (8.77) for myeloperoxidase, and 1.97 μg/g (4.91) for HNL.

Among patients with IBD, markedly increased feces levels of ECP (geometric mean = 74.4 μg/g) and EPX (50.4 μg/g) were found in UC (Fig. 4A). The discriminative capability between UC and normal individuals was, however, somewhat superior for EPX. In CD the increases of ECP (2.38 μg/g) and EPX (2.64 μg/g) were less pronounced but significant ($p < 0.0002$ and $p < 0.0002$). Two of three patients with CC had increased levels of ECP and EPX relative to healthy individuals. Moreover, the levels of ECP and EPX were also significantly higher in UC than in CD ($p < 0.0002$ and $p < 0.0002$).

A similar pattern was observed with myeloperoxidase and HNL (Fig. 4B). Thus, the levels of myeloperoxidase and HNL were markedly increased in UC (817 μg/g and 122 μg/g) and moderately increased in CD (19.2 μg/g [$p < 0.01$] and 5.97 μg/g [$p < 0.05$]). All three patients with CC had increased levels of myeloperoxidase and HNL. Myeloperoxidase was, as shown in Figure 4B, the superior neutrophil marker for discriminating between UC and apparently healthy individuals. Furthermore, the levels of

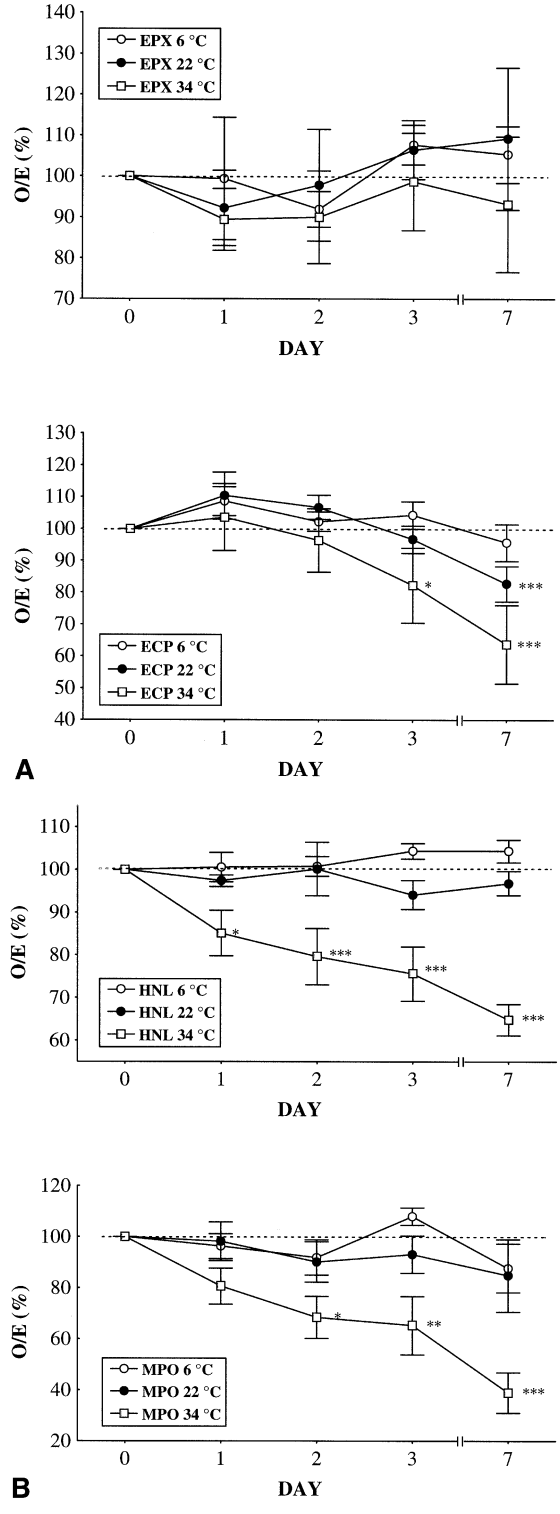

Figure 3. Durability of eosinophil and neutrophil markers in feces. Unprocessed feces samples (n = 5) were after day 0 incubated at +6°C, +22°C, and +34°C for 1, 2, 3, and 7 days. After each of these timepoints samples were processed using extraction buffer and the levels of granulocyte markers were assessed in the supernatants. Data are presented as medians and 25–75% ranges. MPO = myeloperoxidase; O/E (%) = ratio between observed and expected (day 0) levels. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: significant differences between control and experiment data (ANOVA and least significant difference *post hoc* test).

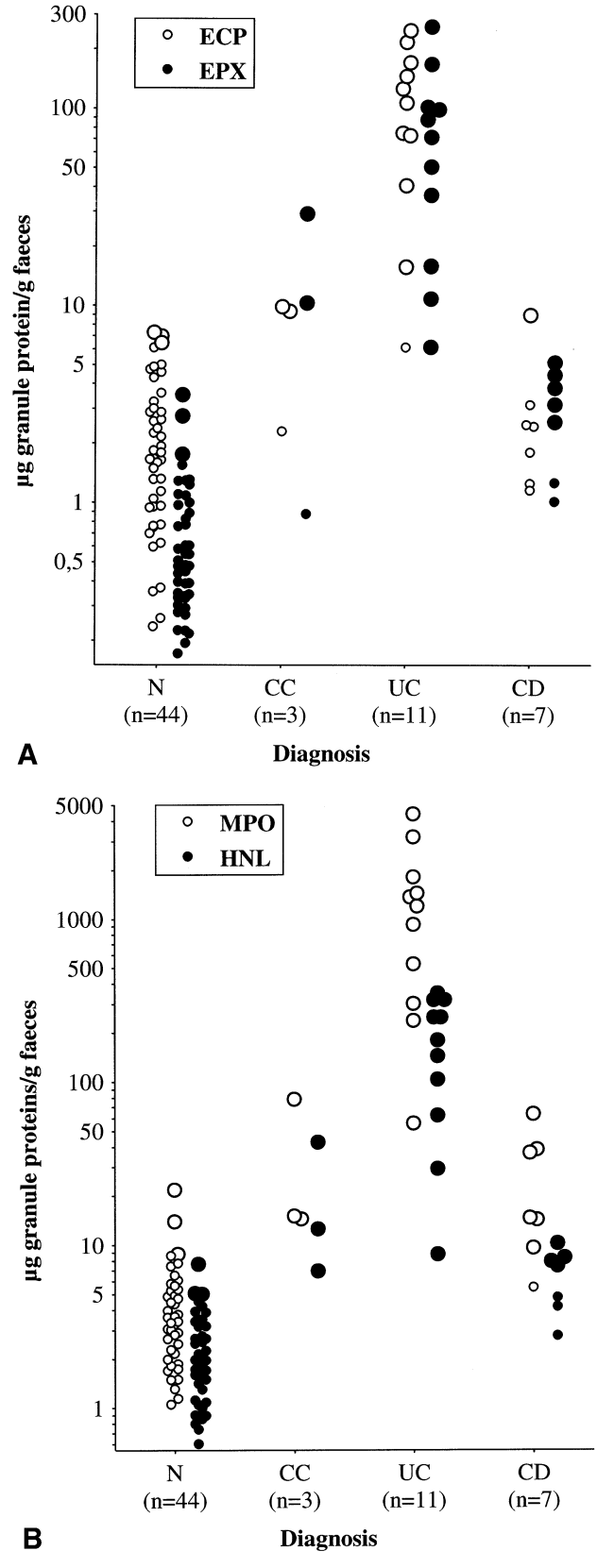

Figure 4. Individual fecal levels of ECP, EPX, myeloperoxidase (MPO), and HNL in patients with UC, CC, or CD compared to normal levels. Enlarged symbols indicate levels higher than the 95th percentile of the normal levels.

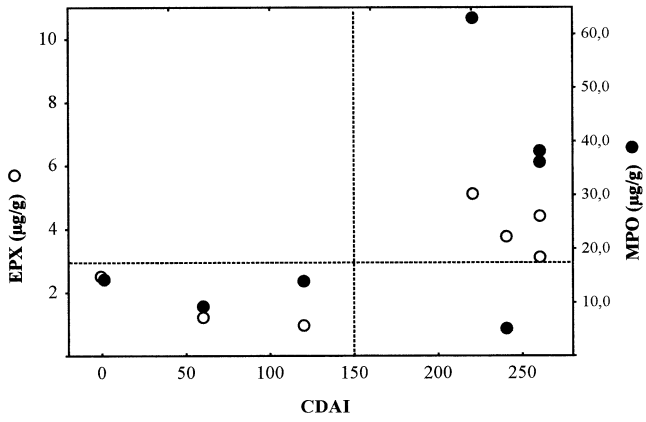

Figure 5. Disease activity in CD in relation to feces levels of EPX and myeloperoxidase (MPO). Patients with CDAI scores of > 150, as indicated by the vertical broken line, were considered to have active disease. The horizontal broken line indicates the upper reference level limits for EPX and myeloperoxidase.

myeloperoxidase and HNL were also significantly higher in UC than in CD ($p < 0.0002$ and $p < 0.0002$).

A clear relationship was observed between disease activity in CD and the levels of EPX and myeloperoxidase in feces (Fig. 5). Thus, all patients with active CD (CDAI > 150) had EPX levels higher than the upper reference limit, whereas patients with inactive disease had normal levels. For myeloperoxidase the only difference in pattern was that one patient with active disease had normal levels. In contrast, the relationship between ECP and HNL and clinical activity was less clear.

Moreover, the relationship between the feces markers and disease activity in UC was less clear; however, a trend toward a relationship between endoscopic score and EPX and myeloperoxidase, respectively, was observed ($R_s = 0.446$, $p = 0.17$; $R_s = 0.560$, $p = 0.07$).

DISCUSSION

We describe here a method for the measurement of the eosinophil granule proteins ECP and EPX and the neutrophil granule proteins myeloperoxidase and HNL in feces. The validation of the method revealed good stability and recovery of these proteins in feces. Three of the proteins—namely, ECP, EPX, and myeloperoxidase—are characterized by their marked cationic charge (pI > 9) (27–29), which makes them very adhesive to various materials and components including feces particles and proteins. Earlier studies on the measurement of ECP and EPX in feces have not taken this fact into account, and measurements were performed on feces just homogenized in water or saline. The aim was therefore to achieve a nearly complete extraction of the markers from feces, and this was accomplished by using the cationic detergent CTAB. This detergent has been successfully used in extraction of granula proteins from eosinophils and neutrophils (30) and also as an important additive in assays [*e.g.*, radioimmunoassays of ECP (31)]. Our results with feces from healthy individuals showed an almost 100 times increased level of ECP in feces after extraction with the CTAB-containing buffer relative to to 0.15-mol/L NaCl. However, samples obtained from IBD patients, which had higher water content, were with respect to EPX and HNL more easily extractable with 0.15-mol/L NaCl than samples obtained from healthy individuals.

The effect of CTAB was in decreasing order less pronounced with myeloperoxidase and EPX, and it was almost noneffective with HNL, which is in agreement with their order of cationic charge.

Our results showed an acceptable recovery of all four granulocyte markers and that they were remarkably stable in feces and processed feces supernatant for at least 3 days at room temperature. This is an important observation because restrictions in handling conditions during and after sampling of feces could be minimized, allowing convenient sampling of feces in, for example, primary care or at home.

In the present study no daily collection of stool samples was performed; however, the long term variation of the markers seems not to be a major obstacle, as indicated by the low CV of the measurements of five samples taken during 8 months from one individual. The consistency of feces is variable and much dependent on the water content. This is particularly true when patients with GI symptoms are studied. In the present study we adjusted the marker levels for the water content of feces. To have a feasible method we avoided freeze drying and instead weighed the feces pellet formed after high speed centrifugation. This procedure allowed us to estimate a semidry weight of feces, which proved to be useful for the adjustment of the marker levels. Adjustment of marker levels for the consistency of feces should be of vital importance at least for the measurement of ECP because most of the feces content of ECP is bound to the particular fraction.

The high levels of the granulocyte markers found in feces from apparently healthy individuals, corresponding to approximately 0.2×10^6 eosinophils/g of feces and 1×10^6 neutrophils/g of feces, indicate that they normally are present at a substantial number in the intestine and cleared into the intestinal lumen.

The method presented here was apart from apparently healthy individuals applied to a limited number of patients with GI inflammations of various severity—namely, patients with UC, CD, or CC. The levels of EPX, ECP, myeloperoxidase, and HNL were significantly increased in both CD and UC. The three patients with CC who are characterized by low grade of inflammation seem also to have increased feces levels of the granulocyte markers. In UC the levels of EPX and myeloperoxidase were very high and clearly discriminating the patients from healthy individuals, which is in agreement with our earlier reports using perfusion fluid of the colon and rectum (32, 33). The results on ECP and EPX partly contrast with other reports (11, 12, 14) where no difference in feces levels between UC and CD

and much lesser degrees of differences between healthy controls and IBD were observed. An explanation for these discrepancies in results could be the use of water or non-optimal extraction buffer for dispersion of feces, as indicated by the overall much lower levels of ECP and EPX in these reports. Moreover, it is unlikely that the arbitrary selection of patients could influence the results in that way, because there were no clear differences in disease activity between UC and CD. We observed a clear relationship between disease activity in CD and feces levels of EPX and myeloperoxidase, whereas such a relation was less clear in the group of UC patients. The latter observation is in agreement with one of our recent reports on segmental perfusion fluid (33). Moreover, a recent controlled treatment study on UC/proctitis (34) showed lack of correlation between myeloperoxidase in the perfusion fluid and endoscopic score before treatment, which however became clearly significant after 28 days of prednisolone treatment. Therefore, the lack of homogeneity of the patients vis-à-vis, for example, treatment regimen in the present study may obscure a relation between disease activity scores and inflammation markers.

In summary, the measurement of fecal levels of the eosinophil granule proteins ECP and EPX and neutrophil granule proteins myeloperoxidase and HNL could be a valuable tool in the examination of the inflammatory status of GI diseases such as IBD. In relation to more invasive methods like endoscopy, this is particularly true when considering the simplicity of collection and storage of feces samples. The method described here takes into account the molecular properties of the granule proteins and the heterogeneity in feces consistency, which is a prerequisite for a valid and reproducible measurement of cationic granule proteins. We suggest, when applied in IBD, that the best eosinophil and neutrophil markers for studying GI inflammation are EPX and myeloperoxidase.

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