Biochemical analysis of enzymic markers of inflammation in rectal biopsies from patients with ulcerative colitis and Crohn's disease

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SUMMARY Rectal biopsies were collected from control subjects, patients with ulcerative colitis both active and quiescent, and from patients with Crohn's disease, both with and without rectal involvement, as judged by histological assessment. Tissue homogenates were assayed for neutrophil (vitamin B₁₂ binding protein, myeloperoxidase, lysozyme) and lymphocyte (5' nucleotidase) selective markers. Patients with acute but not those with quiescent colitis had striking increases of the neutrophil markers. Neither patient group with ulcerative colitis showed a change in the lymphocyte marker enzyme activity. Patients with Crohn's disease involving the rectum showed significant, but less marked, increases in the activity of the neutrophil markers that were found in active ulcerative colitis. Patients with Crohn's disease, not involving the rectum, showed normal or reduced levels of neutrophil markers. Patients with Crohn's disease, both those with and without rectal involvement, had increased activities of the lymphocyte selective marker. This distinguishes this inflammatory response from that of ulcerative colitis and provides further biochemical evidence of abnormalities in apparently uninvolved mucosa from Crohn's patients.

Crohn's disease and ulcerative colitis are the two main inflammatory bowel disorders. Their relation and aetiologies are unknown and it is possible that they share a common cause and that the two conditions represent a spectrum of one disease. Sometimes it is impossible to distinguish one from the other by clinical, histological, or radiological criteria.¹ However, in the majority of cases there are distinct differences.

One of the most striking differences is the histological appearance of the diseased gut. In active ulcerative colitis the lamina propria is diffusely infiltrated by inflammatory cells in which polymorphonuclear leucocytes predominate. In contrast, Crohn's disease involves the whole thickness of the bowel with fewer neutrophils in the inflammatory exudate. The relative paucity of neutrophils in Crohn's disease may be due to the failure of neutrophils to accumulate at a site of inflammation.^{2,3} In view of these findings the biochemical nature of the inflammatory response was studied by assaying rectal mucosa homogenates for neutrophil and lymphocyte selective markers in order to provide quantitative data on the nature of the inflammatory cells.

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Material and methods

A group of 117 patients in whom rectal mucosa histology was required for diagnostic or follow-up purposes were studied. Two biopsies were taken at sigmoidoscopy from adjacent areas in the rectum below the peritoneal reflection. Control biopsies were obtained from 28 patients attending the gastroenterology clinic for investigation of non-specific symptoms in whom no pathology was subsequently found. Any adherent surface exudate was removed. One of the biopsies was placed in formalin for histological examination. They were classified on histological grounds into five groups (Table): 40 patients with Crohn's disease, 21 patients with disease involving the rectum with macroscopic involvement confirmed by either characteristic or compatible histological findings and 19 patients with intestinal Crohn's disease, diagnosed on clinical and radiological grounds but with a normal rectum on histological examination (non-rectal Crohn's disease); 22 patients with active ulcerative colitis with typical histological findings; 27 patients with quiescent ulcerative colitis in whom the acute inflammatory response had resolved but with distorted and

Clinical details of patients studied

	Controls	Crohn's disease	Ulcerative colitis
Number	28	40	49
Age (median and range (yr))	48 (26-76)	44 (18-80)	49 (23-73)
<i>Disease activity</i>			
Inactive	—	30	27
Active	—	10	22
<i>Radiological extent of disease</i>			
Ulcerative colitis:			
rectum	—	—	24
left side	—	—	15
total colonic involvement	—	—	10
Crohn's disease:			
(a) { small bowel		8	—
ileocolonic		11	—
(b) colonic rectal		21	—
<i>Previous drug treatment</i>			
Prednisolone		13	10
Salazopyrine		4	17
Prednisolone and salazopyrine		5	14
None		18	8

(a) = non-rectal Crohn's disease.
(b) = rectal Crohn's disease.

atrophic glandular architecture. The other biopsy was processed for enzyme analysis, by immediately placing it in 3 ml of ice cold sucrose (0.3 M) containing disodium EDTA (1 mM pH 7.4) and ethanol (22 mM) (sucrose medium). The biopsy was then finely mixed in a Polytron homogeniser (Kiner-matica GmbH, Lucerne, Switzerland) for 15 s at a speed setting 2 and disrupted by 10 strokes of loose-fitting (type A) and 10 of a tight-fitting (type B) pestle in a Dounce homogeniser (Kontes Glass Co, Vineland, New Jersey, USA). The homogenate was coded and stored at -20°C for subsequent enzyme analysis.

Unsaturated vitamin B₁₂ binding protein⁴

A sample (0.5 ml) was pipetted into a 10 ml centrifuge tube and 1 ml of [⁵⁷Co] cyanocobalamin 2.22 ng/l (Amersham International) added. The tube was mixed and left to stand at room temperature for 30 min. Two ml of a charcoal suspension, prepared by mixing 3 g of charcoal and 600 mg bovine serum albumin in 120 ml water, was then added to the tube. After mixing and allowing to stand for a further 10 min, the tube was centrifuged at 4°C at 700 g for 10 min in a bench centrifuge. The supernatant was removed from each tube and counted in a Beckman 7000 gamma counter. Results were compared with a standard of 2.5 ml water plus 1 ml ⁵⁷Co-cyanocobalamin solution, without added charcoal reagent, and with a blank of 0.5 ml water plus 1 ml ⁵⁷Co-cyanocobalamin solution with 2 ml of charcoal reagent. Results were expressed as pg vitamin B₁₂ binding protein/mg tissue protein.

Lysozyme (EC 3.2.1.27).⁵

The reaction was performed at 37°C in a Perkin Elmer 557 dual beam spectrophotometer. Substrate (2.5 ml) *Micrococcus lysodeikticus* (Sigma Chemical Co), 250 mg/l in 0.67 M phosphate buffer pH 6.0 was preincubated at 37°C for 2-3 min. Homogenate, 0.1 ml, was added and the reaction was followed with a blank of substrate and sucrose medium only. The reaction was recorded at an absorbance of 405 nm, when the reaction was linear with respect to time; this was achieved after approximately 2 min. Tissue lysozyme was calculated by comparison of sample absorbance with a standard curve of pure lysozyme (Sigma) in phosphate buffer, and expressed as µg/mg tissue protein.

Myeloperoxidase (EC.1.11.1.7).⁶

This activity was measured colorimetrically with O-dianisidine as oxygen acceptor. An aliquot, 0.1 ml, of suitably diluted homogenate was incubated at 37°C for up to 10 min with 1 ml reagent containing H₂O₂ (0.08 mM) and O-dianisidine (0.32 mM) in 0.1 M citrate-NaOH buffer pH 5.5. The reaction was stopped by the addition of 1 ml 350 ml/l perchloric acid and the absorbance of the oxidised dye was measured at 560 nm in a Gilford 300 N spectrophotometer. Activity was expressed as mUnits, where 1 unit corresponds to the conversion of 1 µmol substrate/min.

Lymphocyte selective enzyme

5'Nucleotidase (EC 3.1.3.5).⁷ This enzyme was assayed radiochemically by addition of 0.1 ml suitably diluted sample to 0.5 ml freshly prepared substrate containing 0.12 mM 2,³[H]-adenosine monophosphate (Amersham International) in piperazine-HCl buffer (0.06 M) and incubated at 37°C for up to 60 min. The reaction was stopped by adding 0.25 ml of zinc sulphate (0.25 M) and 0.25 ml of freshly filtered barium hydroxide (0.25 M). After standing for 30 min on ice, the mixture was centrifuged at 800 g for 30 min at 4°C. Supernatant, 0.5 ml, was mixed with 3 ml PCS scintillant (Amersham International) and assayed for radioactivity. Using an external standard, a quench correction was found not to be necessary. The enzyme blank consisted of 0.1 ml sucrose medium, in place of sample, treated as above. The standard contained 1.0 ml of the buffered substrate with 1.2 ml distilled water; 0.5 ml aliquots were counted and the enzyme activity calculated.

Protein was determined by a modification of the method of Lowry, Rosebrough, Farr and Randall⁸ with bovine serum albumin as standard. DNA was assayed by the method of Kapuscinski and Skoczylas⁹ with calf-thymus DNA as standard. The

significance of differences between the means of the group of homogenates was determined by one way analysis of variance. All data showed a Gaussian distribution.

Results

NEUTROPHIL SELECTIVE MARKERS (FIG. 1)

Unsaturated vitamin B₁₂ binding protein

The mean level in rectal Crohn's tissue was 217 pg/mg protein and was significantly above that in normal biopsies (88 pg/mg protein). The mean level in biopsies from patients with Crohn's disease with unaffected rectum was 50 pg/mg protein. The mean activity in active ulcerative colitis biopsies was

Fig. 1 Neutrophil selective marker activities in control subjects, patients with active (A) or quiescent (Q) ulcerative colitis or with Crohn's disease involving (RC) or not involving (NRC) the rectum. Results show mean $\pm$ SEM for indicated number of biopsies assayed in duplicate. Statistical significance comparing patient groups with controls: * $p < 0.01$.

386 pg/mg protein which was significantly raised above that of control biopsies. The level in quiescent colitic biopsies was 127 pg/mg protein which was not significantly raised compared to normal biopsies.

Myeloperoxidase

The mean activity in the rectal Crohn's group (68.5 mU/mg protein) was significantly increased compared to controls (42.9 mU/mg protein). The mean activity in the non-rectal Crohn's disease group (53.4 mU/mg protein) did not differ from controls. The mean activity in the active ulcerative colitis group (75.6 mU/mg protein) was significantly raised compared to controls but normal in patients with quiescent colitis (50.2 mU/mg protein).

Lysozyme

The mean activity in rectal Crohn's disease was 2.7 μ g/mg protein which was significantly higher than the activity in normal biopsies (0.66 μ g/mg protein). The mean activity in biopsies from patients with non-rectal Crohn's disease (0.09 μ g/mg protein), was lower than, but not significantly different from, controls. The mean activity in active colitic biopsies (2.5 μ g/ml) was significantly higher than controls. The activity in quiescent colitic biopsies (0.35 μ g/ml) did not differ from controls.

LYMPHOCYTE MARKER ENZYME (FIG. 2)

5' Nucleotidase

The mean activity was raised in rectal Crohn's biopsies (15.4 mU/mg protein) compared to controls (7.9 mU/mg protein). The mean activity was also

Fig. 2 Lymphocyte selective marker enzyme activity in control subjects, patients with active (A) or quiescent (Q) ulcerative colitis or with Crohn's disease involving (RC) or not involving (NRC) the rectum. Results show mean $\pm$ SEM for indicated numbers of biopsies assayed in duplicate. Statistical significance comparing patient groups with controls: * $p < 0.05$.

raised in Crohn's disease even when the rectum was histologically normal (14.9 mU/mg protein). There was no increase in the active or quiescent ulcerative colitis groups (mean 8.7 and 8.4 mU/mg protein, respectively) compared with controls.

Protein

There was no difference in protein expressed as mg/mg DNA in any of the groups compared to control values. The mean ($\pm$ SE) for control was 0.51 ± 0.16 , rectal Crohn's disease 0.36 ± 0.01 , non-rectal Crohn's disease 0.25 ± 0.15 , active ulcerative colitis 0.35 ± 0.01 and quiescent ulcerative colitis 0.48 ± 0.22 .

Discussion

These results show that patients with rectal Crohn's disease and active ulcerative colitis have increased levels of vitamin B₁₂ binding protein, lysozyme and myeloperoxidase, specific markers for neutrophils, in their rectal biopsy homogenates. In contrast, in Crohn's disease when the rectum is spared, the activity of the neutrophil markers were similar to control values. Kane *et al*¹⁰ found increased blood levels of vitamin B₁₂ binding protein in patients with ulcerative colitis and Crohn's disease. These results were taken to indicate an enlarged granulocyte pool and increased granulocyte turnover in inflammatory bowel disease. Kane and Vincenti¹¹ found a significant increase in vitamin B₁₂ binding protein in active but not in quiescent colitis, compared to controls in rectal biopsy homogenates. These workers however did not distinguish ulcerative from Crohn's colitis and some of their controls had non-rectal Crohn's disease.

Vitamin B₁₂ binding proteins are exclusively localised to the specific granules of neutrophils.¹² Chromatographic analysis of these proteins showed them to be present as equal proportions of transcobalamins I and III.¹² There is considerable evidence, both direct and indirect, that transcobalamins I and III are actively secreted by neutrophil granulocytes and precursors from their promyelocyte stage,¹³ especially when the cells are activated.¹⁴ Vitamin B₁₂ binding proteins may have some bactericidal function as it has been suggested that these proteins can alter the intraluminal colonic bacteria by decreasing vitamin B₁₂ availability.¹⁵

Lysozyme was first described in 1922 and is found in both azurophil and specific granules of neutrophils.¹⁶ Lysozyme hydrolyses the muramic acid-*N*-acetyl-glucosamine bond which is found in the mucopeptide coat of certain bacteria.¹⁷ Kane and Vincenti¹¹ also found increased activities of lysozyme in rectal biopsy homogenates from 11 patients with active colitis. The raised activities

found in rectal Crohn's disease may however not be due solely to neutrophils. Intense lysozyme staining was seen in epithelioid granuloma with immunohistochemical techniques.¹⁸ Monocytes and macrophages also contain smaller amounts of lysozyme¹⁹ and it is possible that these cells also contribute to the raised activities found in Crohn's tissue. The activities found in Crohn's disease with histologically unaffected rectum were lower than controls.

Myeloperoxidase occurs in the azurophil granules of neutrophils and is delivered directly into the phagocytic vacuole when degranulation occurs. In the presence of either I⁻ or Cl⁻, myeloperoxidase can use hydrogen peroxide to kill bacteria.²⁰ Myeloperoxidase deficiency in neutrophils may therefore predispose a patient to infection.²¹ Circulating neutrophil myeloperoxidase activities have however been reported to be normal in patients with inflammatory bowel disease.²²

In the study described here these enzymes were used as biochemical markers to demonstrate the presence of neutrophils in mucosa. They illustrate the importance of these neutrophil enzymes in primary host defence mechanisms. The relatively low activities of the highly specific marker vitamin B₁₂ binding protein in patients with Crohn's disease compared to acute colitis and the low concentrations in patients with non-rectal Crohn's disease is evidence of the failure of neutrophils to accumulate at a site of inflammation. Failure of neutrophils to accumulate at the site of inflammation has been reported to be important in the pathogenesis of Crohn's disease.²³

Studies of rectal, sigmoid and duodenal biopsies from Crohn's disease patients, have shown, irrespective of the endoscopic appearances, increased numbers of macrophages in the lamina propria compared to appropriate controls.²⁴⁻²⁷ In apparently uninvolved jejunum, brush border disaccharidases were reduced but cytoplasmic dipeptidases were unchanged.²⁸ In uninvolved normal rectal mucosa, glucosamine synthetase activity, an enzyme concerned in the biosynthesis of glycoproteins and possibly a measure of cell regeneration was increased.²⁹ Prolyl hydroxylase, was also found to be increased, in rectal mucosa of patients with Crohn's disease many of whom showed no histological evidence of rectal involvement.³⁰ Vasoactive intestinal peptide activities measured by radioimmunoassay and morphological assessment by immunocytochemistry have been found to be raised in rectal biopsies³¹ from patients with Crohn's disease. These studies all indicate that biochemical abnormalities are present in the apparently uninvolved jejunum or rectum of patients with Crohn's disease emphasising the diffuse nature of the disorder.

5'-Nucleotidase is a relatively selective marker enzyme of the plasma membrane.³² Thus its activity in peripheral blood lymphocytes is approximately 50 times that in polymorphs.³³ An abundance is found in lymphocytes, particularly the non T lymphocytes³⁴ and activated macrophages.³⁵ The increased activities found in Crohn's disease probably reflect an increase in lymphocyte or macrophage infiltrate.

From these studies there is a good correlation between the biochemical indices of inflammation and the histological findings in both ulcerative colitis and rectal Crohn's disease. Slightly reduced enzyme activities are found in apparently normal tissue from patients with Crohn's disease. 5'-Nucleotidase activities are increased in Crohn's disease tissue even distant from the site of involvement and may also represent an activated macrophage or lymphocyte infiltration.

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