Mucosal enzymes in human inflammatory bowel disease with reference to neutrophil granulocytes as mediators of tissue injury

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(Received 25 January 1979; accepted 14 May 1979)

Summary
1. Biopsies of rectal mucosa were obtained for histology and enzyme analysis from 32 patients with inflammatory and functional bowel disorders, and the biopsies were classified morphologically as active colitis, quiescent colitis or normal.
2. Supernatant fractions of biopsy homogenates were assayed for their content of the proteolytic enzymes α-chymotrypsin, elastase and cathepsin D, and of protein, unsaturated vitamin B₁₂-binding capacity, lysozyme, myeloperoxidase and N-acetyl-β-glucosaminidase.
3. Mean unsaturated vitamin B₁₂-binding capacity was significantly raised above normal in the active colitic mucosa, and mean lysozyme activity was raised above normal in both active and quiescent mucosae.
4. In active colitic mucosa there was no rise above normal in mean activities of any of the proteolytic enzymes, though a significant fall below normal occurred in mean N-acetyl-β-glucosaminidase activity in the active colitic group.

Key words: colitis, leucocytes, muramidase, peptide hydrolases, regional enteritis, vitamin B₁₂-binding proteins.

Introduction
Infiltration by neutrophil granulocytes of the crypts, surface mucosa and lamina propria is a striking histological feature of active ulcerative colitis, and is a common but less prominent feature in the mucosa of Crohn's disease; indeed, this tissue reaction is shared by 'specific' as well as 'non-specific' inflammatory bowel diseases (Gonzalez-Licea & Yardley, 1966). Evidence from electron-microscopic studies suggests that in colitis neutrophils pass not only between mucosal cells but into them as well (Dobbins, 1975), and are actively degranulating (Gebbers & Otto, 1978). Neutrophil granulocytes have the potential to promote mucosal damage and to mediate other facets of inflammation such as kinin generation (Movat, Steinberg, Habal & Ranadive, 1973) by the release of proteolytic enzymes from their primary granules (Cochrane, 1968; Henson, 1971; Janoff, 1972). The possible role of neutrophil granulocytes as mediators of tissue damage in inflammatory bowel disease has not been fully explored, although one study has demonstrated high levels of collagenase in rectal biopsies of patients with ulcerative colitis (Sturzaker & Hawley, 1975), neutrophils being one potential source of this enzyme (Lazarus, Daniels, Brown, Bladen & Fullmer, 1968).

The principal aim of the present work was to compare proteolytic enzyme activities in rectal biopsies from patients with active and quiescent inflammatory bowel disease, and from normal controls. In addition, we wished to confirm the intestinal wall as a likely source for the raised circulating levels of neutrophil-produced vitamin B₁₂-binding protein and of muramidase (lysozyme) previously described in inflammatory bowel disease.
S. P. Kane and A. C. Vincenti (Kane, Hoffbrand & Neale, 1974). The enzymes studied were the neutral proteases, elastase and \( \alpha \)-chymotrypsin, the acid protease cathepsin D, the bactericidal enzymes myeloperoxidase and lysozyme, and the typical lysosomal enzyme \( N \)-acetyl-\( \beta \)-glucosaminidase. In addition, the tissue levels of \( \alpha \)-amylase were determined to assess possible contamination of the mucosa by pancreatic enzymes. In the human neutrophil granulocyte, elastase, \( \alpha \)-chymotrypsin and myeloperoxidase are localized in the primary granules (Dewald, Rindler-Ludwig, Bretz & Baggiolini, 1975; Kane & Peters, 1975), \( N \)-acetyl-\( \beta \)-glucosaminidase probably resides mainly in a lysosomal sub-population of tertiary granules, lysozyme is distributed between primary and secondary granules, and vitamin \( B_12 \)-binding protein is localized exclusively to the secondary granules (Bretz & Baggiolini, 1974; Kane & Peters, 1975). Catheptic activity is also granule-bound in the cytoplasm of human neutrophils, but has not been precisely localized (Stiles & Fraenkel-Conrat, 1968).

Patients studied

A group of 33 patients seen consecutively as inpatients and outpatients, in whom rectal mucosal histology was required for diagnostic or follow-up purposes, were biopsied. The patients did not undergo any local preparation before sigmoidoscopy. One patient has been excluded since the portion of the rectal biopsy submitted for histology was inadequate for microscopic assessment. The remaining 32 patients fell into a number of groups on the basis of the clinical picture, radiology, previous histology and current sigmoidoscopic appearances. These groups are summarized in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Clinical diagnosis, sigmoidoscopic assessment, age and sex of the 32 subjects whose rectal biopsies were studied histologically and biochemically</th>
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<tbody>
<tr>
<td>Diagnosis</td>
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<tr>
<td>--------------------------------------------</td>
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<tr>
<td>Ulcerative colitis or proctitis</td>
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<tr>
<td></td>
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<tr>
<td>Colo-rectal Crohn’s disease</td>
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<tr>
<td>Ulcerative colitis or proctitis</td>
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<tr>
<td>Ileal Crohn’s disease</td>
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<tr>
<td>Colonic Crohn’s disease</td>
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<tr>
<td>Irritable bowel</td>
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<td>Diverticular disease</td>
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proteins and any undisrupted cytoplasmic organelles from a precipitate of intact cell nuclei and particulate debris such as fibrous tissue. The supernatant fractions of the homogenized biopsies were separated for storage at -20°C until the time of assay.

Repeated freezing and thawing of the samples was considered an adequate stimulus for disruption of the subcellular organelles of the homogenized mucosal and inflammatory cells. Indeed, sonication of a small group of supernatant samples did not increase their vitamin B₁₂-binding protein content. Triton X-100 was added to the assay systems for myeloperoxidase, N-acetyl-β-glucosaminidase and cathepsin D to ensure maximal solubilization of granule proteins, but was found to reduce the activity of α-chymotrypsin and elastase and was therefore excluded from these assays. Goodman, Kent & Truelove (1977) used gentamicin (100 μg/ml) to prevent bacterial growth and enzyme activity in their rectal mucosal glucosamine synthetase assay, but antibiotics were not included in any of our assays. Only one assay, that for cathepsin D, involved an incubation of greater than 1 h, and we found that addition of gentamicin did not reduce enzyme levels.

Activities of all the enzymes measured have been expressed in units of activity/mg of supernatant protein, the protein content of the fractions being determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

The spectrophotometer used for assays of lysozyme, α-chymotrypsin, elastase, α-amylase and cathepsin D was a Pye Unicam SP.1700. A Unicam SP.8000 was used for the assay of myeloperoxidase and a Hitachi Perkin–Elmer model 203 fluorescence spectrophotometer for the assay of N-acetyl-β-glucosaminidase.

The unsaturated vitamin B₁₂-binding capacity of a tissue or biological fluid is a measure of its ability to bind free vitamin B₁₂ added \emph{in vitro}, and is a reflection of the trace amounts of vitamin B₁₂-binding proteins (transcobalaminis) present in that tissue or fluid. The unsaturated vitamin B₁₂-binding capacity levels of the tissue extracts were determined by a modification (Kane et al., 1974) of the rapid charcoal assay of Gottlieb, Lau, Wasserman & Herbert (1965).

Lysozyme (EC 3.2.1.17) was assayed in duplicate at pH 6·2 by the turbidimetric method of Parry, Chandan & Shahani (1965), using killed Micrococcus leisodeikticus (Difco) as substrate.

Myeloperoxidase (EC 1.11.1.17) activity was measured in duplicate by the method of Bretz & Baggiolini (1974), the substrate being 3-chloro-4-nitroaniline, and the incubation was at pH 5·5.

α-Chymotrypsin (EC 3.4.21.1). Benzoyl tyrosine ethyl ester (Fluka) was the substrate for this enzyme, which was assayed at pH 7·2 as described by Gerber, Carson & Hadorn (1974).

Elastase (EC 3.4.21.11). This assay was performed at pH 6·5 as described by Janoff (1969), with N-t-butyloxycarbonyl-L-alanine p-nitrophenyl ester (Sigma) as substrate.

α-Amylase (EC 3.2.1.1). Tissue levels of this enzyme were determined in the presence of 95 μmol/l calcium chloride with the Phasebas amylase kit (Pharmacia) by the method of Ceska, Birath & Brown (1969).

Cathepsin D (EC 3.4.23.5). Acid protease activity was measured in the tissue extracts by the method of Press, Porter & Cebra (1960), which is a modification of the trichloroacetic acid-soluble amino acids when acid-denatured haemoglobin is incubated with the enzyme. The assay was carried out at pH 3·1.

N-Acetyl-β-glucosaminidase (EC 3.2.1.53) was assayed fluorimetrically by the method of Peters, Müller & de Duve (1972), with the artificial substrate 4-methylumbelliferyl-N-acetyl-β-glucosaminide (Koch–Light).

The significance of differences between the means of the three groups of homogenates was determined by Student's \emph{t}-test.

\section*{Results}

\subsection*{Histological classification of biopsies}

There was a good overall agreement between sigmoidoscopic assessment and histological classification. Biopsies from all nine functional bowel disease controls were classed as normal, but the biopsy from the patient with ileal Crohn's disease showed evidence of past colitis. All but one of the relapsed ulcerative and Crohn's colitics had inflammatory activity on biopsy. One patient with proctitis classed clinically as being in relapse had a biopsy showing quiescent proctitis. Of the eight subjects with colitis or proctitis considered on sigmoidoscopic appearances to be in relapse, five had biopsies typical of quiescent colitis, two had normal biopsies, and one had a biopsy showing mild active inflammation. Biopsies from the four patients with active rectal Crohn's disease showed comparable degrees of acute inflammatory infiltration to that in biopsies from active ulcer-
TABLE 2. Therapy specific for inflammatory bowel disease being taken by the patients at the time of rectal biopsy

<table>
<thead>
<tr>
<th>Morphological classification of biopsy</th>
<th>No. of patients</th>
<th>Corticosteroids alone (local and/or systemic)</th>
<th>Sulphasalazine alone</th>
<th>Corticosteroids plus Sulphasalazine</th>
<th>No specific therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active colitis</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Quiescent colitis</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>

Fig. 1. Unsaturated vitamin B₁₂-binding capacity in rectal biopsies from patients with active colitis and quiescent colitis and from histologically normal controls. Numbers in groups are shown in parentheses. In this and subsequent Figures, vertical bars = means ± 1 SD; ■ biopsies from Crohn's disease patients.

The patient's specific therapy at the time the biopsy was taken is shown in Table 2.

Unsaturated vitamin B₁₂-binding capacity (Fig. 1). Most rectal biopsies from patients with active ulcerative or Crohn's colitis contained considerable amounts of vitamin B₁₂-binding protein (mean binding capacity, 367 pg/mg of protein). This differed significantly (t = 2.73; 0.02 > P > 0.01) from the mean of 16 pg/mg for the normal biopsies, but the mean value of 32 pg/mg for the quiescent group did not differ significantly either from the mean binding capacity of the normal group (t = 0.93) or from that of the active group (t = 1.97).

Lysozyme (Fig. 2). The mean activity in the active colitic biopsies (7.91 µg/mg of protein) was significantly raised above that in normal biopsies (2.19 µg/mg of protein; t = 4.05; P < 0.001). The mean lysozyme content of quiescent colitic biopsies (7.18 µg/mg of protein) did not differ significantly from that of the active colitic group (t = 0.38), but showed a significant rise above the mean of the normal group (t = 3.43; 0.01 > P > 0.001). Paneth cell metaplasia occurred in biopsies with both high and low lysozyme activity (Fig. 2).
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Myeloperoxidase (Fig. 3). The mean content of this enzyme in active colitic biopsies (6.45 units/mg of protein) was significantly raised above that of quiescent colitic biopsies (1.33 units/mg of protein; \( t = 2.69; 0.02 > P > 0.01 \)), but was not significantly different from that of the normal control group (4.15 units/mg of protein; \( t = 1.27 \)).

α-Chymotrypsin (Fig. 4). There were no significant differences between the mean activities of α-chymotrypsin in any of the three groups of biopsies studied.

Elastase (Fig. 5). The mean elastase activity of the active colitic biopsies was 11.25 nmol min\(^{-1}\) mg\(^{-1}\) of protein, which was significantly lower \( (t = 2.45; 0.05 > P > 0.02) \) than the mean elastase activity of 15.3 nmol min\(^{-1}\) mg\(^{-1}\) of protein of the normal biopsies. The mean activity of the quiescent colitic biopsies was 13.2 nmol min\(^{-1}\) mg\(^{-1}\) of protein, which did not differ significantly from that of the active group \( (t = 1.05) \) nor that of the normal group \( (t = 1.14) \).

Cathepsin D (Fig. 6). This enzyme was only assayed in the tissue extracts from 11 active colitics and 10 normal controls. The mean activities of the two groups were 321 and 322 nmol of ‘tyrosine equivalent’ h\(^{-1}\) mg\(^{-1}\) of protein respectively and did not differ significantly \( (t = 0.007) \).

N-Acetyl-β-glucosaminidase (Fig. 7). As with elastase, the activity of this enzyme was significantly lower in the active colitis group than in the normal group \( (t = 2.14; 0.05 > P > 0.02) \), the means for the groups being 3.34 and 4.17 units/mg of protein. The mean activity of the quiescent colitis group was 4.07 units/mg of protein, which
did not differ significantly from that of the active group ($t = 1.5$) nor from that of the normal group ($t = 0.18$).

There was no correlation between $\alpha$-amylase activity and the activities of $\alpha$-chymotrypsin ($r = 0.08; \text{N.S.}$) or elastase ($r = 0.05; \text{N.S.}$).

Discussion

These findings show that the neutrophil granulocyte infiltration of the rectal mucosa which occurs in ulcerative colitis and Crohn's colitis is mirrored by raised levels of some granulocyte constituents in mucosal homogenates. Most marked of these is the rise in the unsaturated vitamin $B_{12}$-binding capacity of the mucosa, a rise which pinpoints the inflamed bowel wall as the possible source of the increased concentrations of granulocyte-secreted vitamin $B_{12}$-binding proteins in the sera of patients with active inflammatory bowel disease (Kane et al., 1974). In contrast, assay of mucosal neutral and acid proteases fails to show enhanced enzyme activity in those biopsies where granulocyte activity can be both visualized histologically and demonstrated biochemically.

In addition to the rise in vitamin $B_{12}$-binding protein, there is enhanced lysozyme activity in the inflamed mucosa. The role of lysozyme in inflammatory bowel disease was first investigated by Meyer, Gellhorn, Lehrman, Steinberg & Prudden (1948), who found raised levels of the enzyme in the stool of patients with ulcerative colitis. Subsequent work (Hiatt, Engle, Flood & Karush, 1952) demonstrated a correlation between the granulocyte content of rectal mucus and faecal lysozyme levels in ulcerative colitis, but recently Klass & Neale (1978) have shown that faecal lysozyme extraction is related more to diarrhoea than to colonic inflammation. Lysozyme, though secreted actively by blood and tissue monocytes (Gordon, Todd & Cohn, 1974; McClelland & Van Furth, 1975) and released by dying neutrophil granulocytes (Fink & Finch, 1968; McClelland & Van Furth, 1975), is also found in high concentrations in other types of cell and tissue (Mason & Taylor, 1975). The Paneth cell contains large amounts of this enzyme (Peeters & Vantrappen, 1975) and immuno-histochemical staining for
lysozyme in biopsy material from patients with inflammatory bowel diseases (Klockars, Reitamo, Reitamo & Möller, 1977) showed the enzyme to be present in metaphasic Paneth cells in the colonic crypts, as well as in infiltrating granulocytes and in epithelioid cells of giant cell granulomata. These authors also found that some mucosal crypt cells in both active and quiescent colitis stained positively for lysozyme, whereas normal mucosal crypt cells did not. This finding corresponds to our biochemical study on mucosal homogenates, where lysozyme levels were raised in both active and quiescent colitic mucosa regardless of whether or not there was Paneth cell metaplasia. Although Klockars et al. (1977) found no lysozyme-containing cells in normal rectal mucosa, McClelland, Shearman, Lai A Fat & Van Furth (1976) demonstrated synthesis of lysozyme in vitro by three of 15 normal rectal biopsies, as well as by three of seven active colitic and two of five quiescent colitic mucosae. This accords with the overlap in lysozyme content in the three groups studied in the present work.

The relative contributions of granulocytes, monocytes, Paneth cells and crypt epithelial cells to the rise in serum lysozyme found in some cases of inflammatory bowel disease is far from clear. In an earlier paper (Kane et al., 1974), we did not attempt to distinguish between patients with ulcerative colitis and Crohn’s disease in a group of 21 patients with inflammatory bowel disease shown to have a mean serum lysozyme level raised significantly compared with controls. Subsequent authors (Dronfield & Langman, 1975; Falchuk, Perrotto & Isselbacher, 1975a, b; Founder, Avella, McCallum & Misiewicz, 1975; Mallas, Terry, Asquith & Cooke, 1976; Peeters, Vantrappen & Geboes, 1976) have shown raised serum lysozyme levels in Crohn’s disease, whereas a raised lysozyme level in ulcerative colitis is demonstrable by some methods but not by others (Klass & Neale, 1978). In no series, except that of Falchuk et al. (1975b), has there been any suggestion that serum lysozyme estimation has any value in discriminating between normal subjects, those with ulcerative colitis, and those with Crohn’s disease, and in several reports (Pruzanski & Marcon, 1975; Johansson & Ursing, 1976) the mean serum lysozyme level in Crohn’s patients has not been significantly raised above the mean level in control groups.

Myeloperoxidase levels in rectal mucosa bore a less clear relation to inflammatory infiltration. Levels in quiescent colitic mucosa were, however, significantly lower than those in active colitic mucosa. Neutrophil granulocytes are not an exclusive source of myeloperoxidase, which can also be found in the granules of eosinophilic leucocytes (Archer & Hirsch, 1963) and monocytes (Nicholls, Bainton & Farquhar, 1971). It is possible that slight eosinophilic infiltration may have contributed to the high myeloperoxidase levels in some of the normal biopsies, although several of the ‘quiescent’ biopsies showed similar infiltration.

Aside from their role as tissue markers of infiltration by neutrophil granulocytes and perhaps other inflammatory cells, transcobalamin, lysozyme and myeloperoxidase may all have important antibacterial functions in the inflamed colitic mucosa. The position of the vitamin B₁₂-binding proteins in this field remains most tenuous, but it has been suggested that these proteins can alter the intraluminal colonic flora by decreasing vitamin B₁₂ availability (Gullberg, 1974). The lysis of bacterial cell walls by lysozyme, which has been the subject of considerable study (Chipman & Sharon, 1969), probably takes place both within the phagocytic vacuoles of granulocytes and monocytes, and in the extracellular environment as the result of cell death or active secretion (McClelland & Van Furth, 1975). The granule-bound lysozyme of Paneth cells may give them a similar antimicrobial function to that of granulocytes and monocytes (Erlandsen & Chase, 1972). Myeloperoxidase too plays a well-established part in the intracellular killing both of bacteria (Klebanoff, 1968) and of Candida species (Lehrer, 1975).

A possible role for proteolytic enzymes in the pathogenesis of the colitic lesion has been considered in the past, but has not previously been investigated on tissue extracts. Studies on proteases in faeces of colitic patients have been inconclusive (Warren & Sommers, 1950; Stoughton, 1952; Fleming, Smith & Hendrix, 1966). Why has the present study failed to show any increase in tissue levels of the proteolytic enzymes known to be contained within neutrophil granulocytes? The lack of correlation of α-chymotrypsin or elastase levels with α-amylase activity makes it unlikely that there was significant contamination of the biopsies with blood-borne or luminal proteolytic enzymes of pancreatic origin. A more likely explanation is that enzyme activity has been dampened down by protease inhibitors, in particular α₁-antitrypsin and α₂-macroglobulin. The tissue homogenate is of course a crude system in which to study enzyme activity, tissue levels reflecting the balance between synthesis, catabolism, luminal loss of enzyme, and
inhibitor activity. It is possible that in the acid micro-environment around an inflammatory cell from which enzymes have been exocytosed, proteolysis may take place, unimpeded by these inhibitors, which are mainly effective at neutral pH. In such an environment, however, the neutral proteases would be relatively ineffective while acid proteases (e.g., cathepsin D) might have more potent activity (Janoff, 1972). α₁-Antitrypsin specifically binds and inhibits elastase, trypsin and α-chymotrypsin (Meyer, Bieth & Metais, 1975; Ohlsson & Olsson, 1975), while α₂-macroglobulin entraps and inhibits all classes of endopeptidases including cathepsins, elastase and α-chymotrypsin (Barrett & Starkey, 1973). A rise in mucosal collagenase was demonstrated by Sturzaker & Hawley (1975) in ulcerative colitis. This enzyme is unaffected by α₁-antitrypsin but is completely inhibited by α₂-macroglobulin, suggesting that this latter inhibitor does not play a major part in protease inhibition in the colitic mucosa. Apart from the major circulating inhibitors, local anti-proteases may actually be derived from neutrophil granulocytes themselves (Prokopowicz, 1968).

Apart from inhibition by anti-proteases, the lack of rise of protease levels in the inflamed colitic mucosa could be due in part to a major contribution to enzyme activity in the tissue extracts from the colonic mucosal cells themselves. This certainly seems possible for elastase, where the mean level in inflamed mucosa was significantly lower than that in normal mucosa, in parallel with a fall in N-acetyl-β-glucosaminidase. This latter enzyme is a lysosomal marker in many tissues, and it is of considerable interest that Danovitch, Gallucci & Shora (1972) found falls in the levels of the lysosomal enzymes arylsulphatase and β-glucuronidase in the inflamed mucosae of ulcerative colitics which compared with controls. The lysosomes of normal colonic epithelial cells may then contain both N-acetyl-β-glucosaminidase and an enzyme with esterase activity against the synthetic elastase substrate N-t-BOC-L-alanine-p-nitrophenyl ester, although further studies would be required to show whether or not this esterase has specific protease activity.

There is thus no support from the present studies in vitro for the suggestion that neutrophil polymorph infiltration of the colonic mucosa promotes chronic tissue damage (Kane, 1976). Indeed, those granulocyte-derived proteins which have here been demonstrated in excess in the inflamed colitic mucosa are those with a potential for protection rather than destruction. We have, however, confirmed the presence in the mucosal cells themselves of several biochemical lesions of uncertain significance: firstly, the raised lysozyme content of the quiescent colitic mucosa and, secondly, the lowered lysosomal enzyme content of the inflamed colitic mucosa.

Acknowledgments

We thank the Trustees of the Charing Cross Hospital Clinical Research Committee for financial support, Dr I. M. Murray-Lyon and Dr T. J. Peters for advice and encouragement, and Miss Susan Tippell for secretarial assistance.

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