

Gene expression of group II phospholipase A2 in intestine in ulcerative colitis

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Abstract

Background—It has been suggested that phospholipase A2 (PLA2) has an essential role in the pathogenesis of inflammatory bowel diseases.

Aims—This study aimed at identifying cells in intestinal and mesenteric tissue samples that might express group II phospholipase A2 (PLA2-II) at the mRNA and enzyme protein levels in patients with ulcerative colitis.

Patients and tissue samples—Tissue samples were obtained from the intestine, mesentery, skeletal muscle, and subcutaneous fat of six patients who underwent panproctocolectomy for severe ulcerative colitis. Mucosal biopsy specimens were obtained from the colon of another group of six patients with ulcerative colitis during routine diagnostic colonoscopies. Tissues from six patients without intestinal inflammatory diseases served as controls.

Methods—Tissue samples were studied by light microscopy, immunohistochemistry for PLA2-II enzyme protein, and in situ hybridisation and northern hybridisation for PLA2-II mRNA.

Results—PLA2-II mRNA and PLA2-II protein were detected in metaplastic Paneth cells in six patients and in the columnar epithelial cells of colonic mucosa in four out of six patients with active ulcerative colitis. Positive findings were less numerous in patients with mild ulcerative colitis. Only two out of six control patients had a weak positive signal for PLA2-II mRNA and one of these two patients had a weak positive immunoreaction for PLA2-II in columnar epithelial cells in the colonic mucosa. None of the control patients had metaplastic Paneth cells.

Conclusions—Metaplastic Paneth cells and colonic epithelial cells synthesise PLA2-II in ulcerative colitis. The activity of the PLA2-II synthesis seems to be related to the degree of inflammation in the diseased bowel.

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Keywords: inflammatory bowel disease, ulcerative colitis, phospholipase A2, in situ hybridisation, northern blotting.

Phospholipase A2 (PLA2) is a lipolytic enzyme that catalyses the hydrolysis of the fatty acyl ester bond at the sn-2 position of glycerophospholipids. It is of great interest with

regard to the pathogenesis of inflammation as it is the rate limiting enzyme in the synthesis of eicosanoids and, therefore, considered an important regulator of the inflammatory reaction. In addition, the reaction products of PLA2 (lysophospholipids and non-esterified fatty acids) are cytotoxic substances.¹

Secretory PLA2s are divided into group I and II enzymes (PLA2-I and PLA2-II). PLA2-I is secreted by the exocrine pancreas and serves mainly as a digestive enzyme. PLA2-II has been found by immunohistochemistry in numerous cell types including those of the human gastrointestinal tract.^{2,3} There is evidence indicating an important role for PLA2-II in the pathogenesis of diseases involving inflammation such as infections, sepsis, arthritides, Crohn's disease, and ulcerative colitis.^{4,5} Increased serum concentrations of PLA2-II are found in various inflammatory diseases⁴⁻⁷ including inflammatory bowel diseases.⁸ Minami and coworkers found increased PLA2-II tissue contents and PLA2 activity in colonic mucosa of patients with ulcerative colitis and Crohn's disease.⁹ The studies referred to above give a reason to assume that PLA2-II might be expressed in the inflamed colonic mucosa of patients with ulcerative colitis. However, PLA2-II mRNA was found in the Paneth cells of histologically normal small intestinal mucosa only, whereas no PLA2-II mRNA was detected in samples from normal colonic mucosa by in situ hybridisation.¹⁰

The purpose of this study was to investigate the expression of PLA2-II in tissue samples from the colon and mesenteric lymph nodes of patients with ulcerative colitis. In particular, our aim was to identify cells responsible for the synthesis of PLA2-II in colonic mucosa.

Methods

Samples from the colon, ileum, and mesenteric lymph nodes of six consecutive patients operated on for ulcerative colitis (panproctocolectomy) were studied by light microscopy, PLA2-II immunohistochemistry, and in situ hybridisation and northern hybridisation for PLA2-II mRNA. We also analysed samples from skeletal muscle (the straight muscle of abdomen) and subcutaneous fat in three of these patients. Furthermore, we analysed biopsy specimens from the colonic mucosa (three samples per patient) of six patients with ulcerative colitis undergoing routine colonoscopies. These specimens were studied by the same methods as mentioned above except northern hybridisation.

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Tissue samples were obtained from the colon, ileum and mesenteric lymph nodes during the operation immediately after the resection of the intestine. Samples containing a mucosal area of 20×30 mm and the whole underlying intestinal wall were taken from the most inflamed site of the colonic mucosa, a zone of mild inflammation, and from a macroscopically normal (or closest to normal) part of the colon. Three to four samples of colon and one sample of ileum were taken from each patient at operation. Additional tissue samples were taken from the mesentery and mesenteric lymph nodes (found by inspection and palpation in five out of six cases) close to the inflamed part of the colon. Small muscle and subcutaneous fat samples were taken from the edge of the laparotomy wound. A part of each tissue sample was frozen and stored at -70°C until analysed (pieces of intestinal mucosa, size 5×10 mm, lymph nodes, and mesenteric fat for northern blot analysis). The adjacent larger part of the tissue sample was fixed in 10% formalin and embedded in paraffin wax for histological, immunohistochemical, and in situ hybridisation studies. The Table gives the number of colonic samples analysed from each patient. The inflammatory changes in the colonic samples were graded from 1 to 5 as described earlier.¹¹

Six of the 12 patients had an active disease defined as histological grade of inflammation ≥3 in at least one segment of colon. Histological inflammation grades up to 2 were considered to indicate inactive disease (n=6).

Five of the panproctocolectomy patients (n=6) included in the current study presented with active ulcerative colitis and responded poorly to medical treatment and one patient was operated on for dysplastic changes of colonic mucosa related to ulcerative colitis. Five out of the six panproctocolectomy patients and four out of the six colonoscopy patients with ulcerative colitis received prednisone

treatment at the time of the operation or the colonoscopy, respectively (Table). In addition, seven out of the 12 patients received 5-aminosalicylic acid (patients 1, 4, 5, 6, 8, 10, and 12), and three patients received salazosulfapyridine (patients 2, 9, and 11). The average length of disease history of the patients with ulcerative colitis (n=12, eight men and four women) was 11 years (range 10 months–29 years) and the mean age of the patients was 39 years (range 23–58 years).

Six patients served as controls. Three control patients were operated on for cancer of the colon and three patients underwent diagnostic colonoscopy for mild abdominal symptoms. The colon of these three colonoscopy patients was considered normal. The control samples from the cancer patients were obtained from the resection line of the colon at a distance of at least 30 cm from the site of cancer. In two cases the samples were taken distal and in one case proximal to the site of cancer. There were no signs of intestinal obstruction or any other signs or symptoms of acute disease before the operation. All the control samples were obtained from macroscopically normal colonic mucosa, and were studied by light microscopy, PLA2-II immunohistochemistry, and in situ hybridisation and northern hybridisation for PLA2-II mRNA.

IMMUNOHISTOCHEMISTRY

Sections of formalin fixed paraffin wax embedded tissues were reacted with an IgG fraction of a polyclonal rabbit anti-PLA2-II antiserum.¹² Dewaxed and rehydrated sections were digested with pepsin, (4 mg/ml of 10 mM HCl). The primary antibody was used at a concentration of 0.45 µg/ml. The sections were incubated for 18 hours at +4°C. The primary immunoreaction was localised¹³ with a commercial biotin-avidin based detection system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. For controls, the primary antibody was replaced by preimmune rabbit serum. The sections were counterstained with haematoxylin.

IN SITU HYBRIDISATION

In situ hybridisation was performed on sections of formalin fixed paraffin wax embedded tissues with PLA2-II antisense (test) and sense (control) RNA probes by using a modification of a method described earlier.¹⁰ The 446 base pair cDNA,¹⁴ representing the nucleotides 124–570 of the PLA2-II protein coding area,¹⁵ was subcloned into the HindIII-BamHI site of a pGEM-3Z transcription vector (Promega, Madison, WI, USA). RNA probes were produced with an RNA labelling kit as instructed by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The RNA probes were generated using T7 (antisense probe) or SP6 (sense probe) polymerases after linearisation of the vector with appropriate restriction enzymes. The specific activity of the probes varied from 0.3×10⁸ to 1.0×10⁸ cpm/µg template DNA.

Histological grade of inflammation, immunohistochemistry for PLA2-II, and in situ hybridisation for PLA2-II mRNA in the colonic mucosa of 12 patients with ulcerative colitis and six control patients.

Case No	Light microscopy		Immunohistochemistry			In situ hybridisation			REG
	HG	S	MPC	CEC	S	MPC	CEC	S	
1†	3	(3)	+++	++	(3)	+++	+++	(3)	ReC
2*†	3	(4)	++	+	(4)	++	++	(4)	SiC
3*†	5	(2)	++	++	(2)	+	+	(2)	TrC
4*	4	(2)	+	+	(2)	+	+	(2)	SiC
5*†	4	(2)	+++	–	(2)	++	–	(2)	TrC
6*†	4	(3)	++	–	(3)	++	–	(3)	DeC
7†	2	(3)	++	++	(3)	++	+	(3)	SiC
8*†	2	(3)	+	–	(3)	+	–	(3)	TrC
9†	1	(3)	+	–	(3)	+	–	(3)	CeC
10	2	(3)	+	–	(3)	–	–	(3)	DeC
11	1	(3)	–	–	(3)	–	–	(3)	–
12†	1	(3)	–	–	(3)	–	–	(3)	–
13‡	1	(1)	–	+	(1)	–	+	(1)	DeC
14	1	(1)	–	–	(1)	–	+	(1)	AsC
15	1	(1)	–	–	(1)	–	–	(1)	–
16‡	1	(1)	–	–	(1)	–	–	(1)	–
17‡	1	(1)	–	–	(1)	–	–	(1)	–
18	1	(1)	–	–	(1)	–	–	(1)	–

Six patients had active ulcerative colitis (cases 1 to 6) and six patients had inactive ulcerative colitis (cases 7 to 12). Cases 13 to 18 were control patients. The number of samples analysed is given in parenthesis. HG=Histological grade of inflammation; S=number of samples analysed; MPC=metaplastic Paneth cells; CEC=columnar epithelial cells; +=some positive cells; ++=positive cells common; +++=positive cells dominating; REG=region of colon with most numerous positive cells; ReC=rectal colon; SiC=sigmoid colon; DeC=descending colon; TrC=transverse colon; AsC=ascending colon; CeC=cecal colon.

*Panproctocolectomy. †Prednisone treatment. ‡Cancer of the colon

The hybridisation cocktail contained RNA probes at a concentration of 10^7 cpm/ml. Other modifications to the previous protocol¹⁰ were the addition of an RNase digestion step and a seven day exposure time of the autoradiographic emulsion. RNase digestion was carried out after hot washes for 30 minutes at 37°C. RNase (Sigma Chemicals, St Louis, MO, USA) was dissolved at a concentration of 20 µg/ml in 0.5 M NaCl, 1 mM EDTA, and 10 mM TRIS-HCl, pH 7.5. After autoradiography, the slides were stained with haematoxylin.

NORTHERN HYBRIDISATION

Total RNA was isolated from the tissue samples by homogenisation in guanidine isothiocyanate and centrifugation in a CsCl₂ gradient as described elsewhere.¹⁶ Ten micrograms of total RNA were run on denaturing 1% agarose/formaldehyde gels, stained with ethidium bromide, photographed under UV light, and transferred on to GeneScreen nylon membranes (Du Pont NEN, Boston, MA, USA) according to the manufacturer's instructions. After the transfer, the membranes were rinsed in 2×SSPE (1×SSPE=0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4) and RNA was bound to the membranes with UV crosslinking at 254 nm. The membranes were prehybridised in 5×SSPE, 50% (v/v) deionised formamide, 5×Denhardt's solution,¹⁷ 1% sodium dodecyl sulphate, and 100 µg/ml of denatured herring sperm DNA. Prehybridisation was performed for two hours at +42°C. The probe used was a 0.45 kb cDNA covering the whole protein coding area of PLA2-II cloned into the HindIII-BamHI site of pUC18 plasmid.¹⁴ The insert was labelled with ³²P-dCTP (Amersham, Buckinghamshire, UK) by a random oligonucleotide primer extension¹⁸ to a specific activity of approximately 10^9 cpm/µg. Denatured probe was added to the final concentration of 5×10^5 cpm/ml. Hybridisation was performed for 16 to 24 hours at 42°C. After the hybridisation, the filters were washed twice in 2×SSPE at room temperature for 15 minutes, twice in 2×SSPE, 2% SDS at 65°C for 45 minutes, and finally twice in 0.1×SSPE at room temperature for 15 minutes. Autoradiographic films (Hyperfilm-MP, Amersham, UK) were exposed at -80°C and developed as instructed by the film manufacturer.

ETHICS

The study was approved by the local ethics committee and an informed consent was obtained from each patient.

Results

HISTOLOGY

Histological examination showed Paneth cell metaplasia in the inflamed colonic mucosa in 10 out of 12 patients with ulcerative colitis. The amount of metaplastic Paneth cells seemed to increase in parallel with increasing

inflammatory changes of the colonic mucosa. Histological and immunohistological findings including the distribution of Paneth cells were similar in different regions of the colon and varied only according to the degree of inflammation.

Mild inflammatory changes were seen in the tissue samples from the ileum in five out of six panproctocolectomy patients. The mesenteric (n=6), skeletal muscle (n=3), and subcutaneous fat samples (n=3) showed no signs of inflammation. The histological structure in the tissue samples from the colon of six control patients appeared normal by light microscopy.

IMMUNOHISTOCHEMISTRY

PLA2-II was detected by immunohistochemistry in metaplastic Paneth cells of inflamed colonic mucosa in all six patients who had active ulcerative colitis, and in four out of six patients with inactive ulcerative colitis (Table). The Paneth cells were located mainly in the bottom of the mucosal crypts and gave an intense immunohistochemical reaction for PLA2-II. PLA2-II was found by immunohistochemistry also in the columnar epithelial cells of the inflamed colonic mucosa in four out of six patients with active ulcerative colitis and in one out of six patients with inactive disease (Table). The distribution and number of positive Paneth and columnar epithelial cells seemed to be related to the degree of inflammation in the respective region of the colon. Positive cells were found in any inflamed region of the colon. The immunohistochemical staining intensity of the columnar epithelial cells varied widely. The staining was clear in all cases considered positive but not as strong as the staining in the metaplastic Paneth cells. The immunostaining for PLA2-II in Paneth cells covered the whole cell, whereas the staining in columnar epithelial cells was most intense in the apical cytoplasm. The staining was occasionally restricted to the luminal surface of the columnar epithelial cells. Colonic glands with positive cells were often found adjacent to glands containing no positive cells. Figures 1A and 1B illustrate a positive immunoreaction in Paneth cells and columnar epithelial cells. In addition, immunoreactivity was found occasionally in vascular wall structures of intestinal submucosa. Paneth cells with immunoreactive PLA2-II were found in all samples from the ileum (n=6). No immunoreaction for PLA2-II was found in samples from the mesentery (n=6), skeletal muscle (n=3), or subcutaneous fat (n=3).

There were no metaplastic Paneth cells in the samples of the colonic mucosa from the six control patients. Only one case out of six control patients presented a weak positive immunoreaction for PLA2-II in the columnar epithelial cells of colonic mucosa (Table).

IN SITU HYBRIDISATION

PLA2-II mRNA was detected by in situ hybridisation in metaplastic Paneth cells of the inflamed colonic mucosa in all six patients with

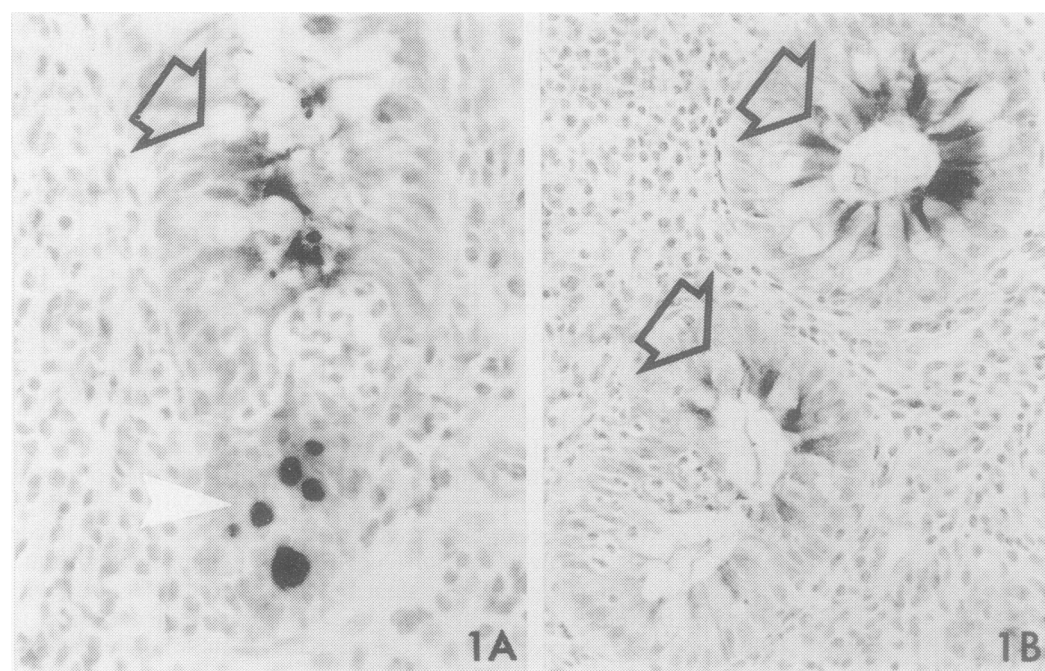


Figure 1: PLA2-II immunoreaction in the colonic mucosa of a patient with active ulcerative colitis (patient 1). Positive findings are present in metaplastic Paneth cells (white arrow) at the bottom of a colonic gland (A). A somewhat weaker immunoreaction (open arrows) is seen also in the apical part of the gland cells (A and B). The reaction is weaker in the lower gland (B) than in the upper gland, although there are infiltrating leukocytes in the lower gland indicating active inflammation. (Anti-PLA2-II antibody, avidin-biotin complex method, haematoxylin counterstaining, original magnification $\times 300$.)

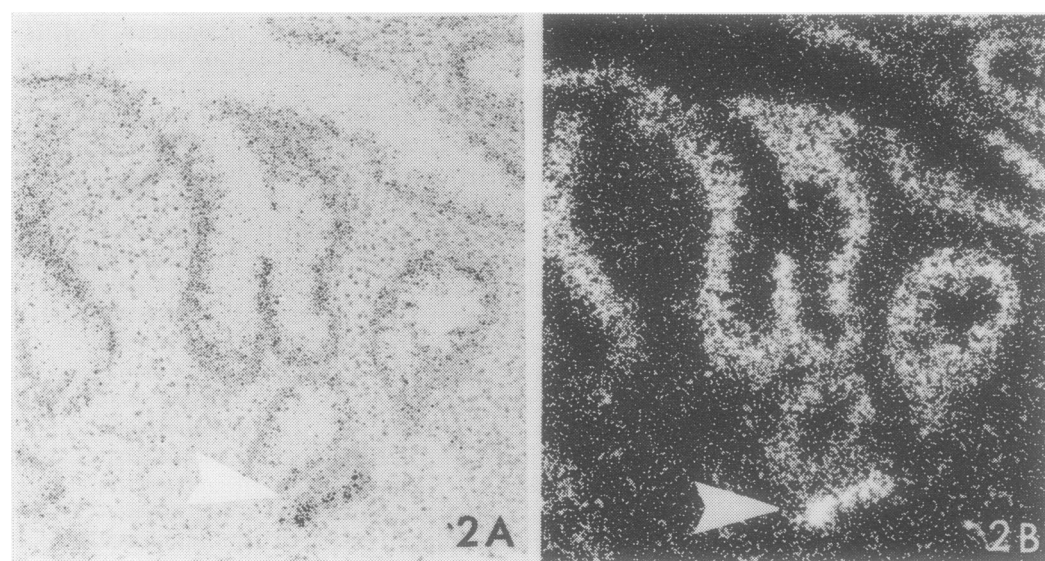


Figure 2: In situ hybridisation for PLA2-II mRNA in colonic mucosa. The tissue sample is from a patient with active ulcerative colitis (patient 1). The autoradiographic grains are localised on metaplastic Paneth cells (white arrows in A and B). A weaker but clear signal is present on the glandular epithelium. (A bright field; B dark field; haematoxylin and eosin, original magnification $\times 120$.)

active ulcerative colitis and in columnar epithelial cells in four out of six patients with active ulcerative colitis (Table). The distribution of the PLA2-II mRNA positive cells in the colonic mucosa was very similar to the distribution of immunohistochemically positive cells as described in the previous section. The intensity of the in situ hybridisation signal seemed to be related to the degree of inflammation and not to any specific region of the colon. Figures 2 and 3 illustrate positive findings in Paneth cells and columnar epithelial cells. PLA2-II mRNA was detected by in situ hybridisation in the Paneth cells of the

ileal mucosa in all patients studied ($n=6$). In addition, PLA2-II mRNA was detected in the epithelial cells of ileum in one out of six patients. The tissue samples from the mesentery ($n=6$) including lymph nodes contained no PLA2-II mRNA, and the same negative result was found in samples from skeletal muscle ($n=3$) and subcutaneous fat ($n=3$) studied by in situ hybridisation.

There were no metaplastic Paneth cells in the colonic mucosa of control patients. We noted a weak positive signal in the columnar epithelial cells in two out of six control patients studied by in situ hybridisation (Table).

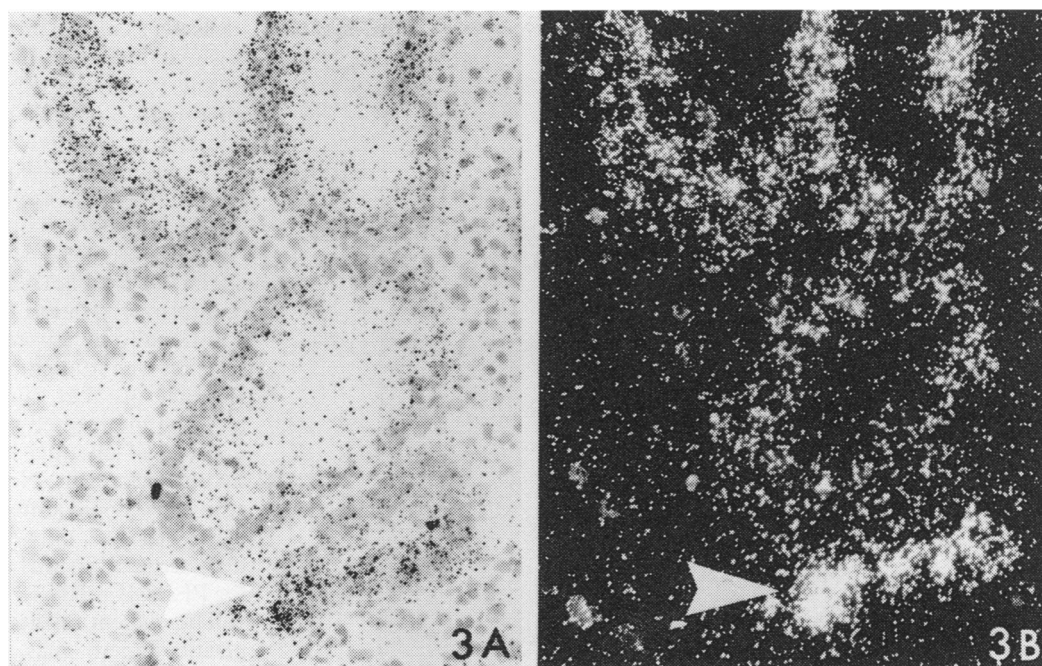


Figure 3: Higher magnification of the section shown in Fig 2. The PLA2-II mRNA signals are on the Paneth cells (white arrow) and columnar epithelial cells of colonic mucosa. (A bright field; B dark field; haematoxylin and eosin, original magnification $\times 300$.)

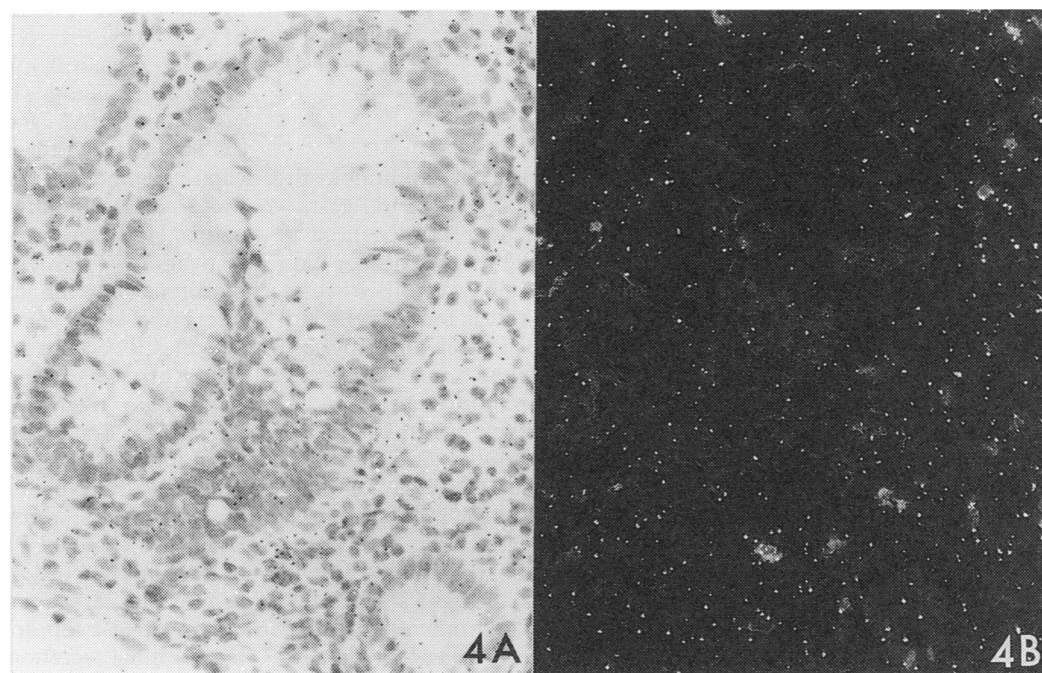


Figure 4: In situ hybridisation with a negative control (sense) probe. The target tissue is in an adjacent section to the one shown in Fig 3. Notice the absence of the hybridisation signal. (A bright field; B dark field; haematoxylin and eosin, original magnification $\times 300$.)

NORTHERN HYBRIDISATION

PLA2-II mRNA was detected by northern hybridisation in tissue samples from mucosa of the colon in five out of six panproctocolectomy patients with ulcerative colitis (Fig 5). The five positive findings were in samples from colonic mucosa that had an inflammatory activity score ranging from 2 to 5 on histological examination. No PLA2-II mRNA was detected in colonic samples in four control patients as studied by northern hybridisation

(Fig 5). Two control samples were excluded from the northern blot analysis because of disintegration of the RNA in these samples. The mRNA detected in the positive samples was 0.9 kb in size, as expected for PLA2-II mRNA.¹⁰

The samples from the mesentery and mesenteric lymph nodes of five panproctocolectomy patients showed no detectable PLA2-II mRNA as studied by northern hybridisation.

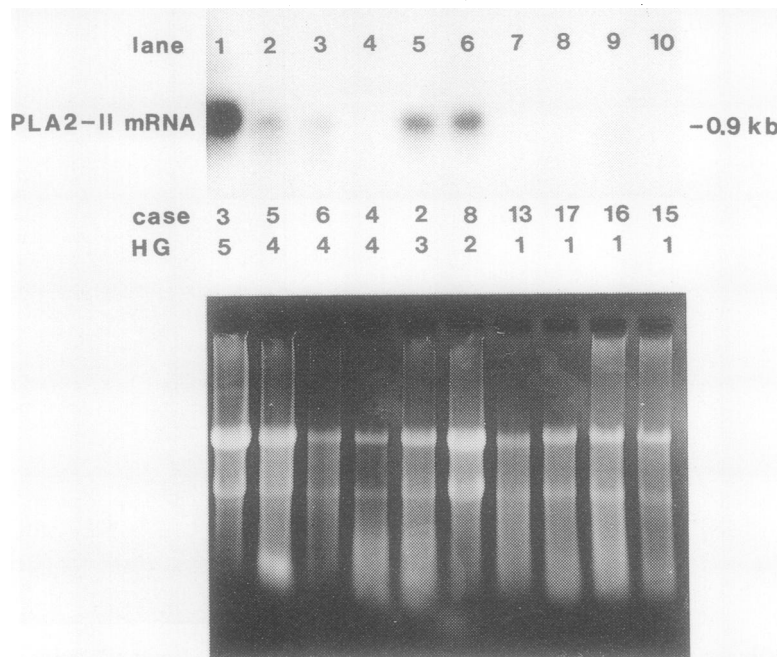


Figure 5: PLA2-II mRNA (upper panel) and corresponding total RNA (lower panel) in samples of colonic mucosa as analysed by northern hybridisation. The RNA samples were from six panproctocolectomy patients with ulcerative colitis (lanes 1 to 6) and four control patients (lanes 7 to 10). The histological grade of colonic mucosal inflammation (HG) at the corresponding site of the sample is given as a number below the case identification number for each patient. Each lane was loaded with 10 µg RNA and the autoradiograph was exposed for seven days.

Discussion

PLA2-II has been found by immunohistochemistry in the epithelial cells of the gastrointestinal tract. The enzyme was localised in Paneth cells and in the brush border and cytoplasm of enterocytes.² In another study, immunoreactive PLA2-II was found in Paneth cell secretory granules but not in any other cell type of the gastrointestinal tract.³ Increased tissue contents of immunoreactive PLA2-II and increased PLA2 activity were reported in colonic mucosa of patients with ulcerative colitis and Crohn's disease.⁹ These findings suggest a role for PLA2 in the pathogenesis of inflammatory bowel diseases.

Northern hybridisation has disclosed PLA2-II mRNA in several human tissues: inflamed synovial tissue, cells of peritoneal exudate,¹⁵ tonsil, kidney and rheumatoid synovial tissue.¹⁹ However, the cells responsible for the synthesis of PLA2-II in these tissues are unknown. Recently, expression of PLA2-II in the human gastrointestinal tract was studied in our laboratory by northern hybridisation and in situ hybridisation. We found that PLA2-II is synthesised and stored by Paneth cells whereas other cell types of the gastrointestinal tract were incapable of synthesis of this enzyme in normal tissue.¹⁰ In another study, higher amounts of PLA2-II mRNA were found by northern hybridisation in the ileal mucosa of two patients with Crohn's disease than in controls.²⁰ To our knowledge, gene expression of intestinal PLA2-II has not been studied earlier at the mRNA level in ulcerative colitis. In the current study, we found PLA2-II mRNA in the colonic mucosa in all patients with active

ulcerative colitis. In addition to the expected positive finding in metaplastic Paneth cells, we found PLA2-II mRNA in the epithelium of inflamed colonic mucosa in four out of six patients with active disease. We also noticed that mesenteric tissue (including mesenteric lymph nodes), skeletal muscle, and subcutaneous fat were devoid of PLA2-II mRNA. All samples were studied by immunohistochemistry for PLA2-II enzyme protein and by in situ hybridisation for PLA2-II mRNA, and additionally, an adjacent part of the same tissue samples was studied by northern hybridisation. Results of the current northern analysis must be interpreted with considerable caution because of unequal loading of RNA into the hybridisation gels. Nevertheless, in the current study, we could differentiate positive samples from negative ones and we found that the detected mRNA was 0.9 kb in size as expected for PLA2-II mRNA.¹⁰

The current results indicate that both metaplastic Paneth cells and columnar epithelial cells of colonic mucosa synthesise PLA2-II at the site of inflammation in patients with active ulcerative colitis. Paneth cells seem to be the main source of PLA2-II as illustrated in the current study at both enzyme protein and mRNA levels. The activation of the synthesis of PLA2-II seems to take place in concert with the increasing inflammatory activity of colonic mucosa in ulcerative colitis, which supports the view that PLA2-II may play a part in the pathogenesis of ulcerative colitis. So far we cannot define the specific role of PLA2-II in the inflamed colonic mucosa in ulcerative colitis. However, there is room for speculation. Activation of PLA2-II synthesis may cause harmful effects on the mucosa by generating free radicals or causing release of lysosomal enzymes.²¹⁻²² On the other hand, PLA2-II might protect the mucosa against invasion of pathogenic microbes or play an immunodefensive part mediated by chemotaxis.²³⁻²⁵ Immunoreactive PLA2-II has been found in the brush border of the luminal surface epithelium of the duodenum.² In the current study, we detected PLA2-II immunoreactivity in the luminal border of colonic epithelial cells in one out of six cases with active ulcerative colitis. These findings support the idea of intraluminal secretion of PLA2-II. It is feasible to postulate that secretion of PLA2-II into the lumen of the gut might be a host defence mechanism during the active phase of ulcerative colitis.

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