Raised concentrations of platelet activating factor in colonic mucosa of Crohn's disease patients

I Sobhani, S Hochlaf, Y Denizot, C Vissuzaine, Emmanuel Rene, J Benveniste, Migwel M J Lewin, Michel Mignon

Department of
Gastroenterology and
INSERM U.10, Paris,
France
I Sobhani
S Hochlaf
Emmanuel Rene
Migwel M J Lewin
Michel Mignon

INSERM U.200,
Clarmat, France
J Benveniste
Y Denizot

Department of
Pathology, Paris, France
C Vissuzaine

Correspondence to:
I Sobhani, MD, Department
of Gastroenterology, 46 rue
Huchard, CHU Bichat-Claude
Bernard, F 75877 Paris, Cedex
18, France.

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Present address: Y Denizot,
CJS INSERM 88-03, Faculte
de Medecine, 2 Rue du Dr
Marclard, F 87025, Limoges,
Cedex, France.

Abstract

Platelet activating factor (PAF-ACETHER or PAF) and precursors of platelet activating factor were investigated in 26 patients with acute Crohn's disease and in 10 healthy controls. Platelet activating factor, lyso platelet activating factor, and alkyl acyl glycerophosphocholine, were determined in colonic mucosal biopsies in patients with acute Crohn's disease, during prednisolone therapy, and in remission. Biopsy specimens were submitted to histopathology examination and to phospholipid extraction. Platelet activating factor, lyso platelet activating factor, and alkyl acyl glycerophosphocholine were found in patients with acute Crohn's disease and in remission as well as in controls. Whatever the site of the biopsy, the level of platelet activating factor in colonic mucosa was higher (p<0·01) in Crohn's disease than in controls. There was no correlation between the level of colonic PAF-ACETHER and age, sex, Crohn's disease activity index, and biological parameters in sera. Although concentrations of colonic platelet activating factor content were higher (p<0·01) when colonic mucosa displayed cell infiltration, they were neither related to the severity nor the type of inflammatory cells. Platelet activating factor decreases with prednisolone therapy and might return to normal concentrations in quiescent patients. Lyso platelet activating factor and alkyl acyl glycerophosphocholine were not significantly higher in Crohn's disease than in controls. These data suggest that platelet activating factor may be involved in the pathogenesis of Crohn's disease and that it could be used as a marker of the mucosal activity of the disease.
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Crohn's disease is a recurrent inflammatory disorder of unknown aetiology and pathogenesis. Histologically, Crohn's disease is characterised by an important infiltration of proinflammatory cells involving the entire gastrointestinal tract from the mouth to the anus. The mechanisms underlying the inflammatory process are poorly understood. When infiltrated by inflammatory cells, the mucosa is known to generate a variety of inflammatory lipidic mediators[1-3] such as arachidonic acid metabolites – for example, prostaglandins, thromboxane, and leukotrienes – and platelet activating factor (also called platelet activating factor acether).

Platelet activating factor, first described in 1972 as platelet activating factor[4] has been identified as 1-0-alkyl-2-0-acetyl-*sn*-glycero-3-phosphocholine. Platelet activating factor is a lipid mediator derived from membrane phospholipids and involving in hypersensitivity and inflammatory reactions.[5] It is synthetised by numerous human cell types, including neutrophils, monocytes, macrophages, platelets, eosinophils, vascular endothelial cells, and several cancer cell lines including gastric epithelial (HGT1) cells.[6 12] It is capable of inducing inflammatory reactions such as increased vascular permeability, leucocyte adhesion and chemotaxis. Some of the manifestations of septic shock, such as hypotension, haemoconcentration, and gastrointestinal ulceration were reported to be mimicked after intravenous administration of platelet activating factor in rats suggesting that it plays a role in this syndrome.[13-18] At present, little is known about the role of platelet activating factor in ulcerative diseases of the gastrointestinal tract in man, although it has been reported in mucosal biopsy specimens from patients with ulcerative colitis and Crohn's disease.[19-21]

The aim of this study was to determine amounts of platelet activating factor and metabolites (lyso platelet activating factor and alkyl-acyl-glycerophosphocholine). In the intestinal mucosa of patients with Crohn's disease related to disease activity in order to assess the possible role of platelet activating factor in the pathogenesis of Crohn's disease.

Methods

SUBJECTS

Twenty six patients (12 men, 14 women; mean age 28 yr, ranging 30–80) with active Crohn's disease (duration of the disease one to 11 years) located to the colon (15), the ileum (three) or both (eight), were investigated. Their diagnoses were based on Morson's morphologic criteria[22] and the activity of the disease was assessed on the Best index (CDAI) and biological parameters (erythrocyte sedimentation rate (ESR), white blood cell count (WBC), platelets, α_2 globulin).

Ten healthy volunteers (five men, five women; mean age 31 yr, ranging 30–80) were investigated as controls.

COLONOSCOPY

All individuals underwent endoscopy examination of the colon (Olympus CF_{20} HL) under sedation (midazolam, noramidopyrin, trimebutin), after colonic preparation with PEG 4000 (4 l) and before anti-inflammatory or immunosuppressive therapy.

Five of the 26 Crohn's disease patients underwent a flexible sigmoidoscopy after four weeks of

1 mg/kg/day prednisolone therapy. A third flexible sigmoidoscopy was performed one month after cessation of all therapy when the disease had been considered in stable remission with no symptoms of recurrence.

Biopsy specimens were taken from non-ulcerated areas in right and left sides of the colon when Crohn's lesions were present and from macroscopically normal mucosa in control subjects and in the five Crohn's disease patients after healing.

HISTOPATHOLOGY

Biopsy specimens were fixed in Bouin's aqueous solution. Sections were stained with haematoxylin and eosin. Mucosal infiltration by inflammatory cells was evaluated as: absent (0), moderate (+), mild (++), intense (+++) by an experienced pathologist who was blinded to the results of platelet activating factor.

PLATELET ACTIVATING FACTOR ASSAY

Biopsy specimens were transferred into tubes with 80% ethanol as previously described for ethanolic platelet activating factor extraction from cells[23] as well as from biopsies[19-21 24] and frozen at $-20°C$ until assayed for platelet activating factor activity. Twenty four hours before platelet activating factor assay, biopsy specimens in 80% ethanol were cut into small pieces (less than 1 mg). Extraction of platelet activating factor was performed during 10 hours at $-4°C$ using a 'roller'. All samples were directly measured using the aggregation of washed rabbit platelets. Rabbit platelets were washed as described previously[25]: aspirinated platelets $(1·6 \times 10^8)$ in 300 μl Tyrode's solution, containing 2·5% gelatin and the ADP scavenger mixture creatine phosphate (1 mM) and creatine phosphokinase (10 IU/ml) were stirred in an aggregometer (Icare, Marseille, France). The aggregating activity of the samples was measured over the linear portion of the calibration curve obtained with 5–50 pg of synthetic platelet activating factor.

ASSAY FOR LYSO PLATELET ACTIVATING FACTOR

Lyso platelet activating factor was chemically acetylated into platelet activating factor, extracted in ethanol and measured as previously described.[23] Briefly, the ethanolic extracts were dried under an air stream, mixed with 200 μl pyridine and acetic anhydride and kept for 18 hours in the dark, at room temperature. The extracts were evaporated and traces of pyridine removed with dichloromethane. Samples recovered with 100 μl of 60% ethanol and then assayed as described above. The amount of lyso platelet activating factor was calculated as the difference between the quantity of platelet activating factor measured after and before acetylation of the samples.

ASSAY FOR ALKYL-ACYL-GLYCEROPHOSPHOCHOLINE (AAGPC)

Alkyl acyl glycerophosphocholine were measured after alkaline hydrolysis and subsequent acetylation.[23] Briefly, aliquots of the dried extracts were treated with 0·03 N NaOH in methanol for two hours at room temperature. The pH was adjusted to 7·0 with 1 N HCl and the mixture was evaporated to dryness. The dried residues were then acetylated as described above and platelet activating factor was assayed. The amount of alkyl acyl glycerophosphocholine was calculated as the difference between the levels of platelet activating factor measured before and after alkaline hydrolysis and acetylation.

CHARACTERISATION OF PLATELET ACTIVATING FACTOR

For further characterisation of the platelet aggregation molecule, platelet activating factor extracted from biopsy specimens from patients with Crohn's disease was pooled, dried and eluted on high performance liquid chromatography.[21 24 26-28] The platelet aggregating activity was eluted from 18 to 21 minutes – that is, with a retention time similar to that of synthetic platelet activating factor. The efficiency of this procedure averaged 80% using $[^3H]$ platelet activating factor as internal standard. In addition to its ADP and aracachidonic acid independent aggregating activity on rabbit platelets, the lipidic material was further characterised as platelet activating factor on the basis of the following criteria: (a) its aggregating activity in the presence of 0·1 mM BN 52021, a specific platelet activating factor receptor antagonist[26]; (b) its aggregating activity after incubation of the samples with 10 μg/ml phospholipase A_2 from hog pancreas and with 100 μg/ml lipase A_1 from *R arrhizus*[27]; (c) its retention time during high performance liquid chromatography analysis using phosphatidylcholine, lysophosphatidylcholine, and synthetic C-18 platelet activating factor as standards.[28]

STATISTICAL ANALYSIS

All values are expressed as mean (standard error of the mean (SEM)). The Mann-Whitney U rank sum test and Spearman rank correlation test were used for statistical evaluation.

The study was approved by the Ethical Committee of the Bichat Hospital.

Results

CLINICAL AND BIOLOGICAL PARAMETERS

In acute Crohn's disease, the Best index (CDAI) was more than 150 in all patients with mean (SEM) 203 (23). Biological parameters were consistent with systemic inflammation (m (SEM)): ESR=51 (5) mm/h, WBC=10322 (632)/dl, platelets=439 (31) 10^3/del and α_2 globulin=8·4 (0·4) g/l. All these parameters were normal in patients with Crohn's disease in remission and in controls.

ENDOSCOPY

Endoscopy examination was normal in all the 10 controls whereas ileal and/or colonic ulcerations
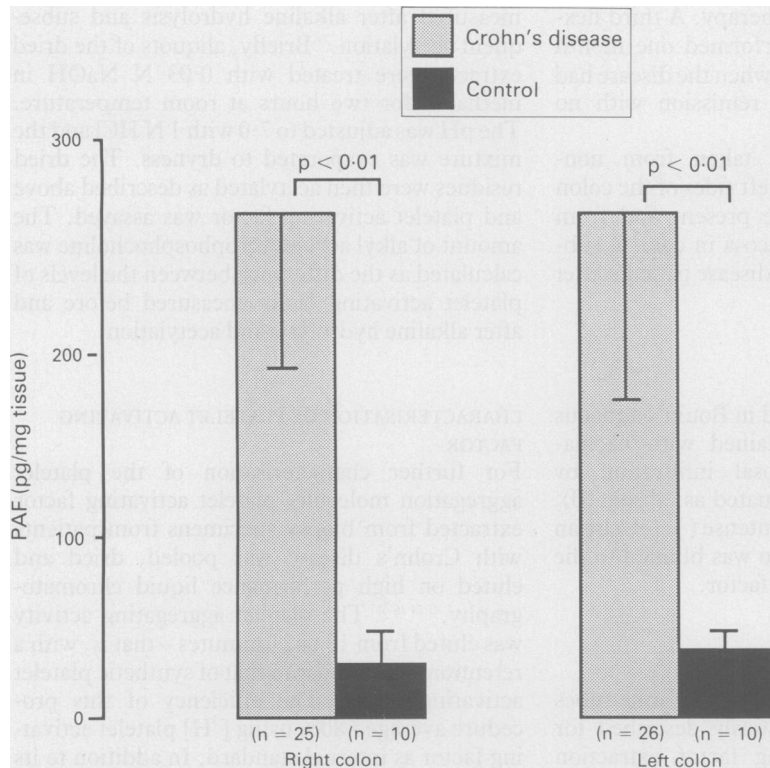
Figure 1: Values are expressed as means (SEM). (n) denotes the number of individuals investigated. PAF=platelet activating factor.

and inflamed mucosa were noted in all Crohn's disease patients before prednisolone therapy. Flexible sigmoidoscopy showed occasional ulceration in the left colon in four of five patients examined during prednisolone therapy and normal mucosa in all patients with Crohn's disease examined in remission.
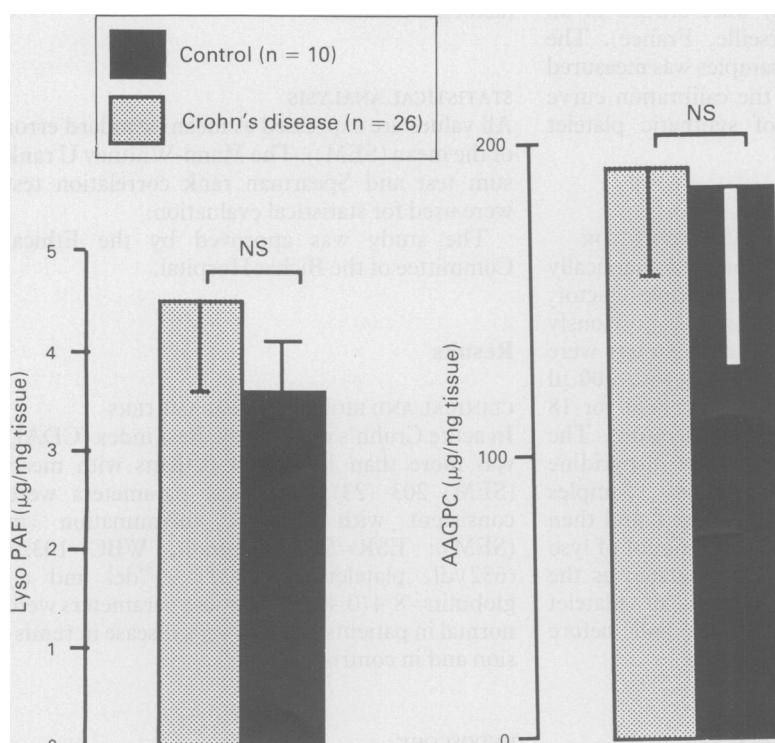

Figure 2: Values are expressed as mean (SEM). (n) denotes the number of individuals investigated; NS: not significant.

HISTOPATHOLOGY

None of the 10 controls had mucosal infiltration by inflammatory cells whereas in patients with active Crohn's disease, inflammatory cells were present in at least one site (left or right) of the colon (26) or in both (23). Mucosal infiltration by inflammatory cells was ranged as absent, moderate, mild, intense in 3, 8, 9, 6 patients respectively in the right site of the colon and in 4, 6, 7, 8 patients respectively in the left site of the colon. After four weeks after prednisolone therapy (five) the inflammation decreased to a lesser extent: two with moderate mucosal inflammatory cell infiltration remained at moderate, two with mild musocal inflammatory cell infiltration returned to normal (absent) and one with intense mucosal inflammatory cell infiltration decreased to mild. The inflammatory cells were absent in the colonic mucosa of Crohn's disease patients examined in remission.

PLATELET ACTIVATING FACTOR IN COLONIC BIOPSIES

The platelet activating factor content of mucosal samples was significantly ($p<0·01$) higher in patients with Crohn's disease than in controls, both in the right (270 (100) v 25 (11) pg/mg net weight) and left colon (280 (110) v 30 (8) pg/mgW) (Fig 1).

Whatever the site of the biopsies, lyso platelet activating factor and alkyl acyl glycerophosphocholine concentrations were not significantly higher in Crohn's disease than in controls (4·5 (1·2) v 3·4 (0·8) μg/mgW and 190 (30) v 180 (60) μg/mgW respectively (Fig 2).

COMPARISON OF PLATELET ACTIVATING FACTOR CONCENTRATIONS WITH CLINICAL, BIOCHEMICAL, AND HISTOLOGICAL PARAMETERS

The mucosal content of platelet activating factor was higher ($p<0·01$) in inflamed than in non-inflamed mucosa, but no significant correlation was observed with the severity of mucosal inflammation (Fig 3). Even the platelet activating factor levels were higher when mucosal histology examination showed granuloma (310 (70) pg/mgW) or mononuclear cells (300 (110) pg/mgW) than polymorphonuclear cells (170 (80) pg/mgW), there was no significant correlation between colonic platelet activating factor content and mucosal infiltration of different type of inflammatory cells (Fig 4).

There was no correlation between the mucosal content of platelet activating factor and the level of age (r': 0·24; p=0·16), sex (r': 0·10; p=0·57), CDAI (r': 0·16; p=0·44), ESR (r': 0·1; p=0·6), platelets (r': 0·14; p=0·48), WBC (r': 0·3; p= 0·1), and $α_2$ globulin (r': 0·3; p=0·09).

PLATELET ACTIVATING FACTOR LEVELS AFTER PREDNISOLONE THERAPY

Five patients with Crohn's disease after treatment with prednisolone underwent flexible sigmoidoscopy. The amount of colonic platelet activating factor content decreased significantly ($p<0·05$) in four and returned to normal values in three patients (Fig 5). There was no significant
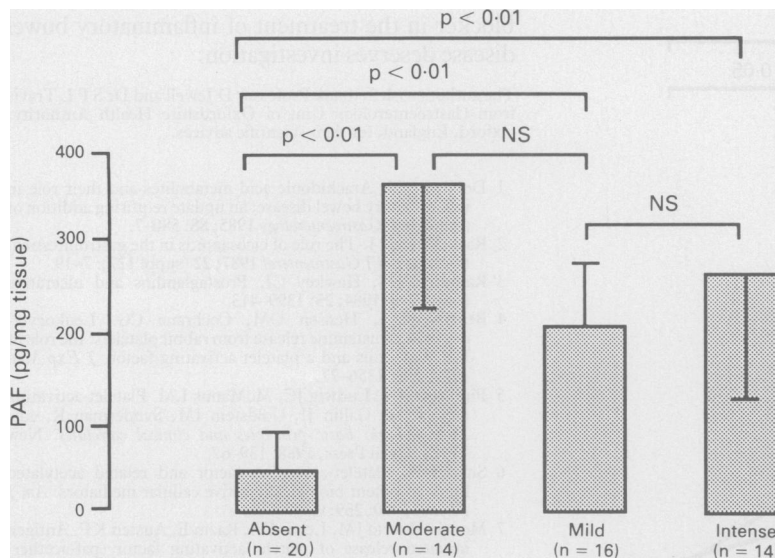
Figure 3: Values are expressed as mean (SEM). (n) denotes the number of individuals investigated; NS: not significant.

difference on mucosal content of platelet activating factor in patients with Crohn's disease in remission than in controls (p=0·07).

Discussion

The results obtained in this study indicate that the mucosal platelet activating factor content is considerably increased in patients with acute Crohn's disease, decreased by glucocorticoid therapy and may return to normal concentrations in quiescent Crohn's disease. Platelet activating factor was extracted from biopsy specimens using ethanolic extraction as previously reported by us[24] and others.[19-20] Biopsy specimens should be conserved in 80% ethanol and frozen at −20°C until assayed for platelet activating factor activity. In these conditions, the platelet activating factor acetylhydrolase activity (the enzyme which cleaves platelet activating factor into the inactive lyso platelet activating factor) which is naturally present in biopsy specimens is inactivated. One freeze treatment did not affect the acetylhydrolase activity. Ethanolic extraction is now a common method of extracting platelet activating factor from various organs including biopsy specimens.[19-21 23 24] In the present study the aggregating activity of materials obtained from patients with Crohn's disease was characterised as

platelet activating factor according to: (i) its retention time from high performance liquid chromatography which was similar to that of synthetic platelet activating factor; (ii) its inhibition by specific platelet activating factor antagonist and phospholipase A2 (PLA2) and no by PLA1.[25-28]

The possibility that platelet activating factor may be involved in Crohn's disease has been suggested by Kald et al.[20] These authors have shown that colonic platelet activating factor content was significantly increased irrespective of colonic inflammation. Our data supported by quantification of mucosal inflammatory cell infiltration, suggest that the increase of platelet activating factor levels in colonic mucosa of Crohn's disease patients are neither related to the severity nor the type of inflammatory cell infiltration. So far, the cellular origin of platelet activating factor from colonic mucosa remains unclear. The lack of correlation between platelet activating factor content and the severity of inflammation in colonic mucosa shown by our present findings, speaks against inflammatory cells as the sole source of platelet activating factor synthesis. In addition platelet activating factor was also found, although to a lesser extent in normal, uninflamed mucosa. The possibility that intestinal epithelial cells may produce platelet activating factor has been suggested by Kald et al.[20] Although the present investigation did not reveal more platelet activating factor in colonic mucosa in patients with Crohn's disease in remission than in controls, this hypothesis could be possible but needs further investigations. Alternatively, the presence of platelet activating factor reflects not only inflammatory cell infiltration, but may also represent a stage of inflammatory amplification in which inflammatory cells are about to be recruited. This hypothesis seems likely, because Crohn's disease patients with inflamed mucosa had a very high mucosal platelet activating factor content that decreased during prednisolone therapy, and sometimes returned to normal levels in remission. It is well known the Crohn's disease is a diffuse lesion of the entire gastrointestinal tract. We have not investigated platelet activating factor content, however, in duodenal or jejunal mucosa of Crohn's disease patients, therefore we have no information as to whether the increase of platelet activating factor levels occurs all along the gastrointestinal tract.

Numerous data have suggested a role of arachidonic acid metabolites in the pathophysiology of various gastrointestinal disorders. For example, enhanced synthesis of protaglandins, thromboxane A_2 and prostacyclin and leukotriene B4 have all been shown in chronic inflammatory bowel diseases.[2 3 29-32] It has been reported that PLA2 activity contributes to the inflammatory process and promotes the formation of arachidonic acid metabolites.[1 33] Of interest, two metabolic steps are involved in platelet activating factor biosynthesis: first, PLA_2 acts on choline-containing membrane alkyl-phospholipids resulting in the production of lyso platelet activating factor – that is, 1-0-alkyl-sn-glycero-3-phosphocholine[11]; and, second, this lyso platelet activating factor compound is acetylated by an
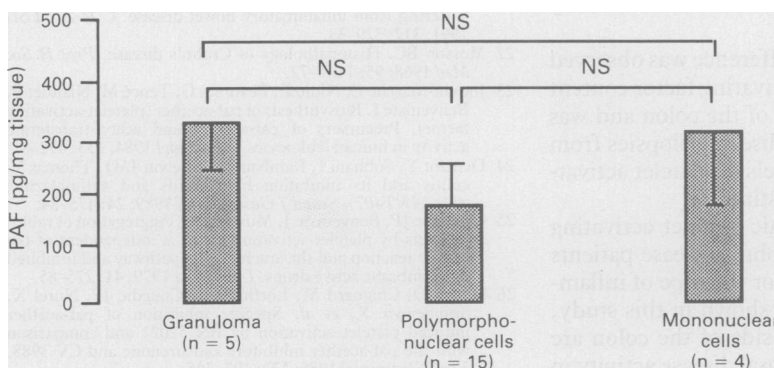

Figure 4: Values are expressed as mean (SEM). (n) denotes the number of individuals investigated; NS: not significant.
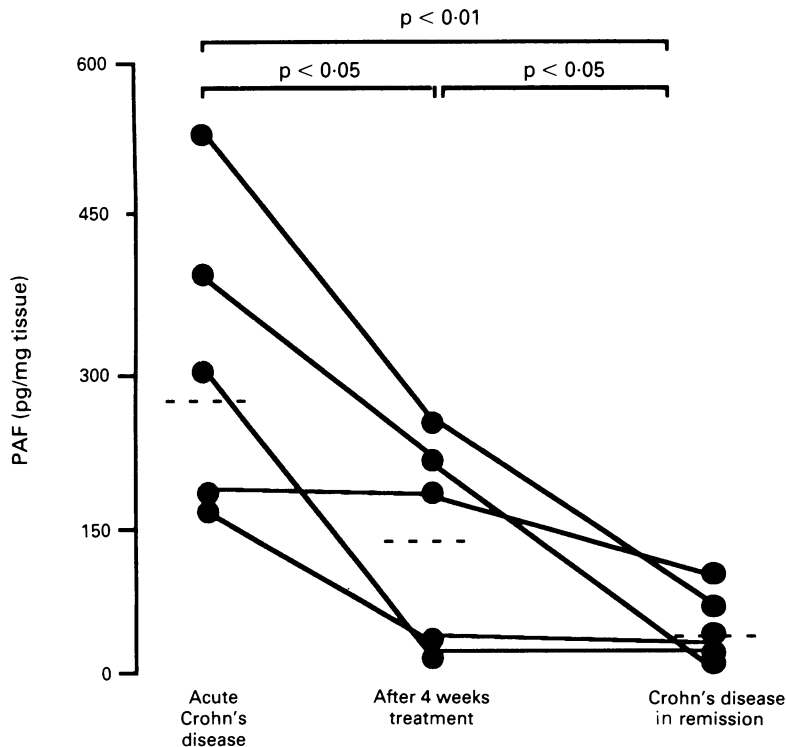
Figure 5: Each point denotes a patient with Crohn's disease with a follow up during prednisolone therapy (four weeks) and in remission (three to five months after prednisolone therapy beginning). (....) denotes the mean value of colonic paf from all five patients.

acetyltransferase to yield a biologically active platelet activating factor molecule.[34] Eliakim et al[19] have reported that prednisolone and 5-aminosalycilate inhibit colonic platelet activating factor synthesis in vitro. The mechanism of this inhibition is poorly understood. As PLA2 has been shown to be increased in Crohn's disease patients,[35 36] it has been postulated that steroids inhibit colonic platelet activating factor synthesis by interfering with PLA_2 activity. The fact that lyso platelet activating factor concentrations are not significantly enhanced in acute Crohn's disease mucosa compared with normal mucosa, however, may suggest that raised platelet activating factor levels are related to an increased acetyltransferase rather than to PLA_2 activity. The role of specific acetyltransferase activity in Crohn's disease patients has not been studied so far, and deserves investigation. It is unknown whether high activity of enzymes is related to change involving the inflammatory cells or both intestinal epithelial and inflammatory cells. Therefore we suggest that platelet activating factor should be considered as a global mucosal activity marker.

Because no significant difference was observed between colonic platelet activating factor content from the left or right sides of the colon and was unrelated to the extent of disease, biopsies from sigmoid may reflect the levels of platelet activating factor in the whole intestine.

In conclusion, the colonic platelet activating factor is raised in acute Crohn's disease patients irrespective of the severity or the type of inflammatory cell infiltration. As shown in this study, two biopsies from the left side of the colon are sufficient to assess the mucosal disease activity in Crohn's disease patients whatever the location of the disease. The role of platelet activating factor

blocker in the treatment of inflammatory bowel disease deserves investigation.

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