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Expression of nitric oxide synthase in inflammatory bowel disease is not affected by corticosteroid treatment

N Leonard, A E Bishop, J M Polak, I C Talbot

Abstract

***Aim*—To examine the effect of corticosteroid treatment on the expression of inducible nitric oxide synthase (iNOS) in the colon of patients with inflammatory bowel disease.**

***Methods*—Four groups of patients were studied: (1) ulcerative colitis treated with high dose corticosteroids (six patients, 10 blocks); (2) ulcerative colitis patients who had never received corticosteroids (10 patients, 16 blocks); (3) Crohn's disease treated with high dose corticosteroids (12 patients, 24 blocks); (4) Non-inflammatory, non-neoplastic controls (four patients, six blocks). Full thickness paraffin sections of colons removed at surgery were immunostained with an antibody raised against the C terminal end of iNOS. Sections were assessed semi-quantitatively for the presence and degree of inflammation and immunoreactivity for nitric oxide synthase.**

***Results*—Cases of ulcerative colitis and Crohn's disease with active inflammation showed strong staining for nitric oxide synthase. The staining was diffuse in ulcerative colitis and patchy in Crohn's disease, in accordance with the distribution of active inflammation. Staining was seen in epithelial cells and was most intense near areas of inflammation such as crypt abscesses. Non-inflamed epithelium showed no immunoreactivity. Treatment with corticosteroids made no difference to the amount of nitric oxide synthase.**

***Conclusions*—Expression of nitric oxide synthase is increased in both ulcerative colitis and Crohn's disease and appears to be unaffected by treatment with corticosteroids. Disease severity necessitated surgery in all the cases included in this study, regardless of whether or not the patients had received long term corticosteroid treatment. It seems therefore that a high level of iNOS expression and, presumably, production of nitric oxide characterise cases which are refractory to clinical treatment; this suggests that specific inhibition of the enzyme may be a useful therapeutic adjunct.**

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Keywords: inducible nitric oxide synthase; inflammatory bowel disease; Crohn's disease; ulcerative colitis; immunocytochemistry

Department of Histopathology, St Mark's Hospital, London WC2, UK
N Leonard
I C Talbot

Department of Histochemistry ICSM, The Hammersmith Hospital, London W12, UK
A E Bishop
J M Polak

Correspondence to:
Dr N Leonard, Department of Histopathology, Royal Victoria Infirmary, Newcastle University, Queen Victoria Road, Newcastle upon Tyne NE1 4LP, UK.

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Nitric oxide (NO) is involved in various physiological processes. It has many different actions at a cellular level, including raising intracellular levels of cGMP, inhibiting enzymes involved in mitochondrial respiration and DNA synthesis, and the formation of reactive oxygen intermediates. In addition to its physiological actions, it has also been implicated in many disease processes. It is reduced in atherosclerotic plaques and pre-eclamptic placentas and increased in neurodegenerative disorders and endotoxic shock.[1]

NO is a gas and has a very short half life. Although measurement of its production is possible, direct visualisation of its cellular sites of production is not. Instead, localisation of nitric oxide synthase (NOS), the enzyme which produces it, is used as an alternative. There are three types of NOS—neural NOS (type 1), found mainly in the brain; endothelial NOS (type 3) produced by endothelial cells; and inducible NOS (iNOS) (type 2) which can be produced by many different cell types. As its name implies, iNOS is an inducible enzyme, and its production can be triggered by circumstances such as infection, injury, or inflammation.[2]

Recent studies have shown increased NO production in inflammatory bowel disease, specifically ulcerative colitis, with some conflicting results in Crohn's disease.[3–8] A major source of this NO appears to be epithelium. Glucocorticoids have been known for some time to inhibit the expression of iNOS.[9] In view of the frequent use of corticosteroids to treat inflammatory bowel disease in the past few decades, these studies on iNOS in inflammatory bowel disease have included treated patients. Thus the possible confounding effect of corticosteroid treatment on iNOS expression in colonic epithelium in a clinical setting has not been evaluated before. The aim of the present study, therefore, was to evaluate iNOS expression in the colon in ulcerative colitis and Crohn's disease and to determine the effect of corticosteroid treatment by comparing samples from treated patients with those taken from patients known not to have received any corticosteroids during the course of their disease.

Methods

Tissue was examined from four different patient groups retrospectively:

(1) Patients with ulcerative colitis who had been treated with high dose steroids (10 blocks from six patients);
(2) Patients with ulcerative colitis who had never received steroids (16 blocks from 10

patients); this group of patients were operated on in the 1950s and 1960s when treatment with corticosteroids was usual but not universal;

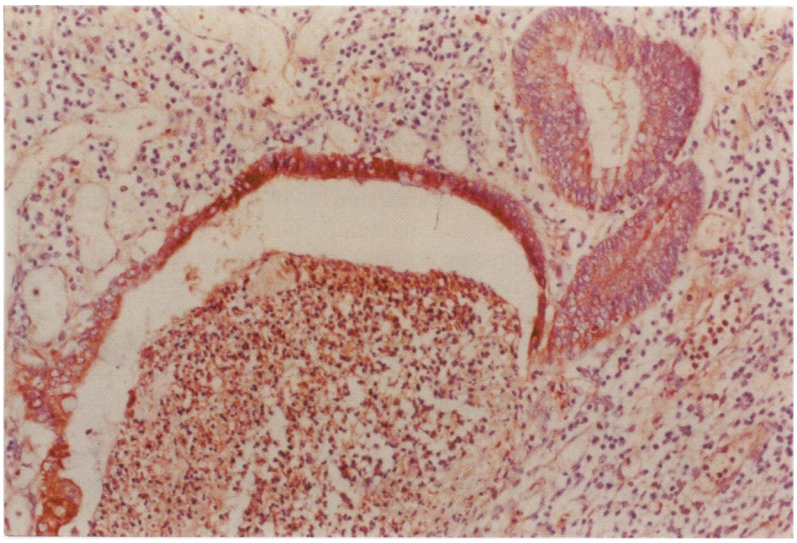

Figure 1 Ulcerative colitis with positive epithelial staining around a crypt abscess. The polymorphonuclear cells in the abscess are also positive.

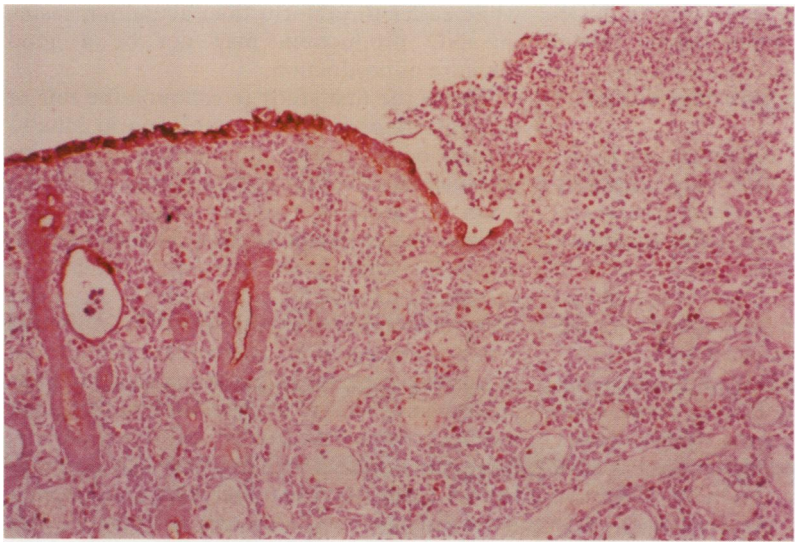

Figure 2 Positive epithelial staining in Crohn's disease adjacent to an area of ulceration.

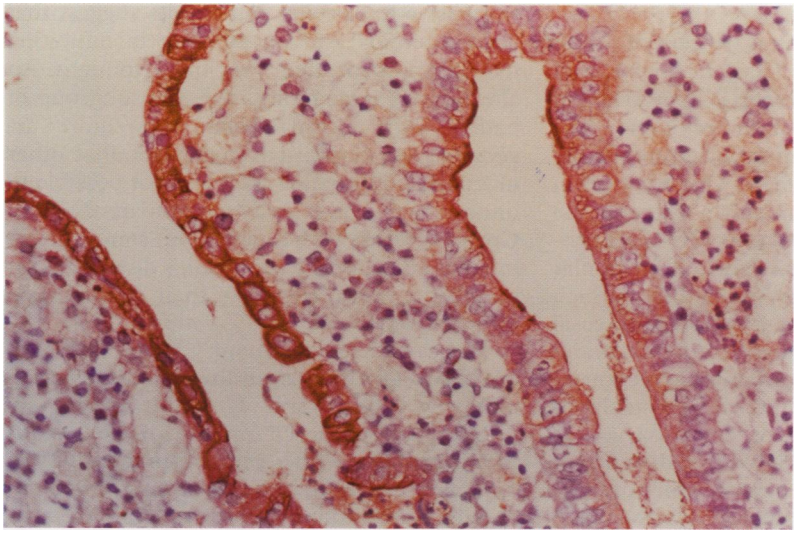

Figure 3 Higher power with positive epithelial staining. The reaction product is located in the cytoplasm. Some cells have deeper staining in the brush border.

(3) Crohn's disease patients treated with steroids (24 blocks from 12 patients);
(4) Non-inflammatory, non-neoplastic controls (six blocks from four patients); two of these patients had idiopathic constipation, one had idiopathic malrotation, and one had a rectal haemangioma.

Patient groups 1 and 3 had received a variety of medical treatments including sulphasalazine and azathioprine. Patient group 2 had received sulphasalazine in some cases but not azathioprine (too early for this). Patient group 4 had no medical treatment.

Full thickness sections of colon removed at surgery had been formalin fixed and paraffin embedded. Where possible there were two blocks from each case, with differing amounts of inflammation in each. The avidin–biotin peroxidase complex method of immunostaining was used.[10] The antibody reacted to the 19 amino acids at the C terminal end of iNOS (1:500; Santa Cruz Biotechnology).

The cases were assessed for the presence and degree of inflammation using the Saverymuttu score.[11] This gives a grade of between 0 and 3, 0 having no or minimal inflammation and 3 having maximum inflammation. All of the controls had a score of 0. Cases with inflammatory bowel disease had scores between 0 and 3.

Immunostaining of iNOS staining was assessed as follows:

Amount of staining: absent = 0; focal = 1; diffuse = 2

Intensity of staining: none = 0; pale = 1; intense = 2

The above values were added together to give an iNOS score between 0 and 4.

Results

Immunostaining was seen in epithelial cells and was most intense near areas of inflammation, such as areas with crypt abscesses (fig 1) and in epithelium adjacent to ulceration (fig 2). Non-inflamed epithelium did not stain with iNOS antibody. The staining was strong when positive and equivocal staining was not seen. The reaction product was located in surface epithelial cells, in the cytoplasm. Some cells showed a deeper area of staining in the brush border (fig 3). The bases of the epithelial crypts did not stain. In addition to epithelial cells, polymorphonuclear cells in abscesses, lamina propria, and blood vessels stained for iNOS, as expected.

The results are illustrated in table 1. Both ulcerative colitis and Crohn's disease cases with active inflammation showed strong iNOS staining. The staining tended to be diffuse in ulcerative colitis and patchy in Crohn's disease. This is related to the different patterns of active inflammation seen in these diseases, with diffuse active inflammation in ulcerative colitis compared with patchy activity in Crohn's disease. Granulomas seen in Crohn's disease did not stain for iNOS. Treatment with corticosteroids in ulcerative colitis made no difference to the amount or intensity of iNOS staining.

The six normal controls showed no iNOS positivity. Cases of ulcerative colitis and

Table 1 Inflammation score compared with nitric oxide synthase (NOS) score

NOS score				
4		XX	CXX UUU	XX CU
3	C	C	CCC CX	CC
2		X	C	CC XU
1		X	X	
0	NNNNN NUUXX CCCCC CCCCC	XX CC		
	0 Inflammation score	1	2	3

C, Crohn's disease; N, control; U, ulcerative colitis, steroids; X, ulcerative colitis, no steroids.

Crohn's disease with minimal chronic inflammation without active inflammation also showed no iNOS staining.

Discussion

Our results confirm those of others[3-8] in showing that there is abnormal expression of iNOS by epithelial cells in inflammatory bowel disease. In addition, we have now shown that treatment with corticosteroids does not affect this expression.

Involvement of NO in inflammatory bowel disease has been suspected only relatively recently. Middleton *et al* showed that rectal dialysates from patients with active ulcerative colitis contained increased nitrate, an end product of NO catabolism.[3] The origin of this nitrate was unknown until two separate studies showed that colonic mucosa from patients with ulcerative colitis contained calcium independent NOS activity.[4 5] This has to come from iNOS, as the other two enzyme types—types 1 and 3—are calcium dependent. Singer *et al* were the first to localise iNOS in ulcerative colitis where, as in the present study, they found iNOS expression in colonic epithelium which is actively inflamed.[6] It is absent from non-inflamed epithelium.

There are many potential triggers of iNOS expression, including infection, injury, and inflammation. The preferential expression of iNOS in the brush border of some cells suggests that NO is being exported out of the cells to the luminal surface. This may be in response to some as yet unknown trigger in the bowel lumen. In small amounts NO has a protective role. It has been shown to have similar functions to mucosal prostaglandins, which protect the mucosa by maintaining mucosal blood flow, stabilising inflammatory cells and increasing mucus and bicarbonate secretion by the epithelium. NO can increase prostaglandins by altering levels of cyclo-oxygenase expression.[12] Several studies have shown that suppression of NO by enzyme inhibitors can make the mucosa more susceptible to injury. Unfortunately, higher levels of NO can also cause tissue damage. Cell damage and death can occur, which could produce the ulceration seen in inflammatory bowel disease.

While several studies have shown consistent results in ulcerative colitis, there have been more conflicting results in patients with Crohn's disease. Boughton-Smith *et al* found significantly increased iNOS activity in ulcerative colitis but not in Crohn's disease or controls.[4] Although Crohn's disease dialysates showed more iNOS activity than controls, the difference was not statistically significant. Rachemilewitz *et al* measured NO production by cultured mucosal explants by measuring the conversion of (^{3}H)-L-arginine to (^{3}H)-L-citrulline.[5] They found that Crohn's disease mucosa produced twice as much NO as ulcerative colitis mucosa, but that iNOS activity was twice as high in ulcerative colitis as in Crohn's disease. Oudherk Pool *et al*, however, found a similar increase in NOS in both ulcerative colitis and Crohn's disease.[13] We have here shown that actively inflammed mucosa in Crohn's disease does express iNOS but that intervening normal mucosa does not. This may account for the smaller amounts of NO/iNOS seen in Crohn's disease in some studies. It is important to note that immunocytochemical measurement of iNOS expression, while easier to perform than actually measuring NO production, may not be a good measure of production.

This is the first study to examine the role of corticosteroids in iNOS expression in inflammatory bowel disease in a group of patients with ulcerative colitis. Although many different drugs are given in active inflammatory bowel disease, it is arguable that corticosteroids, especially when given intravenously, have a very powerful effect. Corticosteroids can affect NO production in several ways.[9] By inhibiting cytokine release they can decrease the amount of positive triggers on iNOS expression. They are also supposed to inhibit iNOS induction at a cellular level.[14] As can be seen from table 1, there was no difference in iNOS expression in steroid treated and non-steroid-treated ulcerative colitis patients. Although it is possible that among patients with ulcerative colitis those who require surgery are a subgroup less responsive to steroids, our results suggest that corticosteroids are less effective in inhibiting iNOS expression than previously thought. An alternative explanation is that the subgroup of patients with ulcerative colitis who require surgery are less responsive to steroids that other ulcerative colitis patients. It was not possible to undertake a similar study to compare iNOS in Crohn's disease with and without steroid treatment as all patients with Crohn's disease had at some time in the course of their disease been given steroids.

The cause of inflammatory bowel disease is unknown, and the reason for the differences in ulcerative colitis and Crohn's disease are unclear. One proposed initiating mechanism is that in patients with a genetic susceptibility to inflammatory bowel disease, once any non-specific inflammation has occurred the immune response is overactive, leading to increased injury and ulceration rather than to

healing without scarring.[15] In this scenario, excessive amounts of NO would contribute to the damage. Excessive epithelial NO has been shown in both ulcerative colitis and Crohn's disease in this study and in previous studies. The effects of excessive NO include cell toxicity and death, which would produce mucosal ulceration. Large amounts of NO could produce muscle dilatation and the toxic megacolon seen in ulcerative colitis.[1 2]

As with many cellular molecules, it can be difficult to distinguish an initiating event from secondary effects. It is of course possible that NO is only one of many secondary mediators stimulated in the ever widening inflammatory cascade. However, this does not diminish the potential therapeutic importance of modulation of NO production. NO has been used as treatment in many different diseases such as the use of NO donors which produce vasodilatation in angina and NO inhalation for chronic pulmonary hypertension. In the gastrointestinal tract NO donors have been combined with non-steroidal anti-inflammatory drugs in the hope that the protective effects of NO would balance the side effects of these agents.[12] NO suppression has been attempted in experimental models of endotoxic shock, a condition where some of the damage is attributed to excessive levels of NO, but equal damage was produced with subnormal levels of NO. To summarise, there appears to be a very fine balance between the protective and destructive effects of NO.

In conclusion, we have shown that iNOS is increased in inflammatory bowel disease and that in patients with ulcerative colitis it does not matter whether or not patients are treated with corticosteroids. Our findings support the contention that blocking the effects of NO may provide a useful therapeutic adjunct.

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