

From The Department of Medicine, Solna  
Karolinska Institutet, Stockholm, Sweden

# **Nitric Oxide and Evaluation of Different Treatments in Experimental Colitis and Inflammatory Bowel Disease**

**Sofie Lundberg**



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*To my family*



You only live once, but if you do it right, once is enough

*~Mae West~*



## ABSTRACT

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of unknown etiology of the gastrointestinal tract. Central features of IBD are increased nitric oxide (NO) generation in the gut lumen and dysfunction in regulation of leukocyte recruitment from the blood stream towards the affected tissue.

This study aimed to investigate NO and the role of collagen-binding  $\alpha 2\beta 1$  integrin in IBD and experimental colitis, with special reference to how NO is synthesised, how NO affects gut motility, how rectal NO levels can be used to identify corticosteroid refractory IBD patients, and to compare anti-integrin treatment to current treatment regimes in IBD.

The results show that expression of inducible NO synthase (iNOS) is increased in inflamed colonic tissue in both animal and man, and could be the biomarker for colonic inflammation, that we today are lacking. The induced overproduction of NO is likely to be responsible for the decreased motility in colitis where NO is suggested to exert a suppressive tone on colonic contractility, which is reversed by blockade of NO synthase. Results show that low (<1000 ppb) initial rectal NO levels upon onset of flare, were distinct to the patient with a steroid-refractory disease, later subjected to colectomy. Thus, rectal NO levels could be a useful biomarker of treatment response in IBD.

An alleviating action of the collagen binding  $\alpha 2\beta 1$  integrin in experimental colitis is demonstrated and suggests that this effect is mediated by inhibition of neutrophil migration and activation. The studies show that anti-integrin treatment through rectal administration of anti- $\alpha 2$  or anti- $\alpha 4$  integrin antibodies reduced clinical and histological signs of colitis in mice. The protective effects against colitis seen after anti-integrin treatment are favourable and have therapeutic potential beyond current treatment regimens with 5-ASA, betamethasone, immunomodulators and cytostatic compounds. Local administration of function-blocking antibodies against integrin  $\alpha 2\beta 1$  may provide novel avenues to treat inflammatory bowel disease.

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## **APPENDIX (paper I-IV)**

## ABBREVIATIONS

The main abbreviations used in this thesis:

5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
ACh	Acetylcholine
AZA	Azathioprine
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
cNOS	Constitutive nitric oxide synthase
DAI	Disease Activity Index
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GCS	Glucocorticosteroids
GI	Gastrointestinal
HBI	Harvey Bradshaw Index
IBD	Inflammatory bowel disease
IL	Interleukin
INF	Interferon
iNOS	Inducible nitric oxide synthase
L-NAME	N <sup>ω</sup> -Nitro-L-arginine methylester
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO <sub>2</sub> <sup>-</sup>	Nitrite
NO <sub>3</sub> <sup>-</sup>	Nitrate
NOS	Nitric oxide synthase
O <sub>2</sub>	Oxygen
ppb	Parts per billion
RT-PCR	Reverse transcriptase-polymerase chain reaction
TNF	Tumour necrosis factor
TTX	Tetrodotoxin
UC	Ulcerative colitis



## INTRODUCTION

### Inflammatory bowel disease

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of unknown etiology of the gastrointestinal tract. The diseases are characterised by periods of remission and exacerbation. Common symptoms associated with active UC and CD are diarrhoea, usually involving discharge of blood and mucus, and abdominal pain. In more severe cases signs of systemic inflammation such as fever, malaise, anorexia, and weight loss may also be present. IBD are furthermore associated with shortening of the colon, motility disturbance and disturbance in the colonic contractile response (Manousos and Salem 1965; Snape, Matarazzo et al. 1980; Oxelmark 2006). In the most severely affected cases which are more or less refractory to medical treatment, surgical resection of the affected bowel may become necessary.

UC and CD are more common in the industrial world than in developing countries but the incidence continue to rise in low-incidence areas such as southern Europe, Asia, and much of the developing world. In the same time, the prevalence and incidence are beginning to stabilize in high-incidence areas such as northern Europe and North America. 1.4 million persons in the United States and 2.2 million persons in Europe suffer from these diseases (Loftus 2004). In Sweden the annual incidence of UC is approximately 13/100,000 with a prevalence of 235/100,000 inhabitants. The annual incidence of CD in Sweden is approximately 8/100,000, with a prevalence of 213/100,000 inhabitants (Tysk and Jarnerot 1992; Lapidus 2006).

### Definition of ulcerative colitis and Crohn's disease

UC and CD are associated with similar symptoms but the diseases differ in several respects. The diagnoses are based on clinical symptoms, endoscopic, histological, or radiological findings (Sleisenger 2002). UC is per definition restricted to the colon (Fig. 1), involving the rectum and to variable extent the colon. The inflammation is typically continuous and restricted to the mucosa. In CD the entire gastrointestinal (GI) tract, from the oral cavity to the anus, can be affected, with preference for the terminal ileum, caecum, and colon. The inflammation is typically discontinuous, with affected segments separated by unaffected mucosa. A classical endoscopic feature is the cobblestone pattern, caused by longitudinal and transverse linear ulcerations of the bowel wall.

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The inflammation is transmural i.e. the inflammation progresses from involving the mucosa, through the submucosa to the muscular layers. This transmural inflammation in CD leads to the development of fistulae and stenosis.

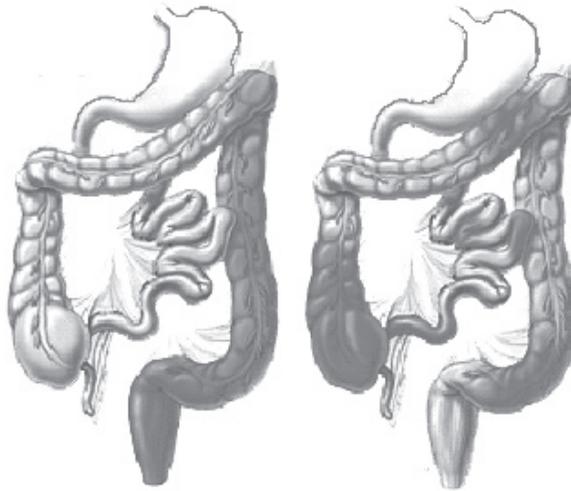


Figure 1. Schematic drawings showing which parts (dark grey) of the gastrointestinal tract that are affected by inflammation in ulcerative colitis (left) and in Crohn's disease (right).

In patients where the inflammation is confined to the rectum and colon it may be impossible to discriminate between UC and CD, which has led to the disease entity called indeterminate colitis. Approximately 10% of patients having colonic IBD are classified as indeterminate colitis (Stewenius, Adnerhill et al. 1995).

A central feature of IBD is dysfunction in regulation of leukocyte recruitment towards the affected tissue. T cells, neutrophils, and monocytes have been shown to serve an important role in modulating immune responses and subsequent tissue damage (Fiocchi 1998; Panes and Granger 1998; van Assche and Rutgeerts 2002; Wen and Fiocchi 2004).

### **Treatments in inflammatory bowel disease**

There are no medical treatments that can cure IBD today. Thus, the main goal is to induce and maintain remission. The current medical treatment of IBD relies upon the use of anti-inflammatory and immunosuppressive drugs with limited specificity, severe side effects and limited long term benefits (Sandborn and Targan 2002; Baert, Vermeire et al. 2004).

### *Treatment of ulcerative colitis*

Sulfasalazine consisting of one 5-aminosalicylic acid (5-ASA) molecule and one sulfapyridine molecule linked together by an azo-binding, was the first drug to induce remission in active UC (Svartz 1942). Sulfasalazine is still on the market but to avoid the side effects associated with the sulfonic part of the drug, new 5-ASA compounds have been developed. There are today several sulfonic free alternatives e.g. mesalazine and olsalazine, proven to be effective for induction and maintenance of remission in UC (Sutherland and MacDonald 2003; Sutherland and Macdonald 2006). These drugs are indicated for first-line treatment of active UC. Glucocorticosteroids (GCS) are efficacious for the treatment of active UC and will often be added to induce remission (Nayar and Rhodes 2004). GCS have however no maintenance benefits in preventing relapse. GCS treatment is also associated with well-known side effects, such as weight-gain, hyperglycemia and diabetes, acne, cutaneous striae, cataract, osteoporosis and mood disorders (Rutgeerts, Löfberg et al. 1994). Immunomodulators such as 6-mercaptopurine (6-MP), azathioprine (AZA) or cyclosporine are options if intravenous steroids fail to induce remission in severe acute UC (Fraser, Orchard et al. 2002; Shibolet, Regushevskaya et al. 2005). Infliximab, a chimeric monoclonal tumour necrosis factor alpha (TNF- $\alpha$ ) antibody, administered as i.v. infusions has recently also proven its usefulness for induction of remission and maintenance treatment of therapy refractory UC (Rutgeerts, Sandborn et al. 2005).

### *Treatment of Crohn's disease*

The role of sulfasalazine and 5-ASA in the induction and maintenance of medically induced remission of CD is limited (Akobeng and Gardener 2005). GCS are used to induce remission in active CD, and AZA, 6-MP or methotrexate can be added for maintenance of remission (Feagan, Fedorak et al. 2000; Nayar and Rhodes 2004). Budesonide (a second generation of GCS) with fewer GCS-associated side effects and enhanced anti-inflammatory activity is a standard drug for ileocolonic CD (Rutgeerts, Löfberg et al. 1994). Infliximab has become the mainstay treatment for both induction and maintenance of remission in refractory luminal and fistulizing CD (Present, Rutgeerts et al. 1999; Cohen, Tsang et al. 2000; Rutgeerts, Van Assche et al. 2004). However, there is a risk of severe opportunistic infections including tuberculosis, which may limit extensive use of TNF- $\alpha$  antibody treatment (Mayordomo, Marengo et al. 2002).

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## Assessment of disease activity in IBD

The need for activity indices originates from the search for objective markers of improvement in controlled clinical trials. First to describe such a disease index for UC was Sidney Truelove who, in 1955 developed a three-grade scale (Truelove and Witts 1955). Truelove's scale, still in use, has been followed by several other numerical indices, including Powell-Tuck Index (Powell-Tuck, Bown et al. 1978) and the Disease Activity Index (DAI) developed by Sutherland (Sutherland, Martin et al. 1987). In 1976, the Crohn's Disease Activity Index (CDAI) was developed on the basis of the American National Cooperative Crohn's Disease Study (Best, Beckett et al. 1976; Best, Beckett et al. 1979). Harvey and Bradshaw later developed Harvey Bradshaw Index (HBI), a simplified version of CDAI that accurately expresses the clinical activity of CD (Harvey and Bradshaw 1980). In 1994 Lichtiger developed a disease activity scale for UC similar to the HBI, called Index of Lichtiger (Lichtiger, Present et al. 1994). These later two indices have made it easier to perform statistical comparisons of the disease activity in UC and CD patients in connection with clinical studies.

## Nitric oxide

In 1980, the biological effects of NO were recognized, initially referred to as endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki (Furchgott and Zawadzki 1980). Six years later two independent research groups, including Ignarro and Murad, demonstrated that EDRF was NO. In 1998 Furchgott, Ignarro and Murad were jointly awarded the Nobel Prize in Physiology or Medicine for their discoveries concerning NO as a signalling molecule in the cardiovascular system. Since then NO has been found to be involved in many processes and diseases throughout the body.

### The chemistry of NO

NO is one of the smallest (molecular weight 30 D) and simplest biologically active molecules present in nature. At room temperature and atmospheric pressure NO is a colourless gas which easily diffuses across bio-logical membranes due to its uncharged and lipophilic nature.

In aqueous solution NO has a half-life in the range of seconds, being dependent on the partial pressure of oxygen ( $O_2$ ), when NO converts primarily to nitrite ( $NO_2^-$ ).  $NO_2^-$  may then subsequently oxidize further to nitrate ( $NO_3^-$ ). In vivo the half-life of NO is even shorter due to presence of scavenger proteins and by the reaction of NO with transition metals, e.g., the iron in oxyhemoglobin to form  $NO_3^-$  and methemoglobin (Doyle and Hoekstra 1981).

### Formation, enzymatic synthesis, and non-enzymatic formation

At high temperatures NO can be formed from molecular nitrogen ( $N_2$ ) and  $O_2$  but in the human body most of the NO is enzymatically derived from the guanidine group of the amino acid L-arginine via an oxidation reaction catalyzed by a family of enzymes referred to as NO synthase (NOS). This reaction (Fig. 2) is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrate and flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), calmoduline (CaM), heme and tetrahydrobiopterin ( $BH_4$ ) as co-factors (Nathan and Xie 1994). There is also a non-enzymatic pathway for NO production in biological systems, a reduction of  $NO_2^-$  to NO (Benjamin, O'Driscoll et al. 1994; Lundberg, Weitzberg et al. 1994). After concentration in the saliva of dietary  $NO_3^-$  (e.g. from green leafy vegetables like lettuce and spinach) and reduction to  $NO_2^-$  by tongue surface bacteria,  $NO_2^-$  is chemically reduced to NO in the acidic conditions of the stomach (Duncan, Dougall et al. 1995; McKnight, Smith et al. 1997). The bacterias involved were characterized in humans 2005 (Doel, Benjamin et al. 2005). This non-enzymatic pathway to form NO seems to be important both in host defence against swallowed pathogens and in gastric physiology (Duncan, Dougall et al. 1995).

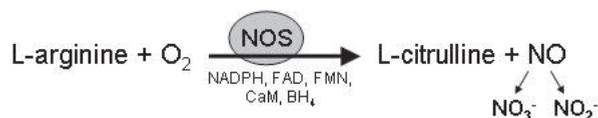


Figure 2. Synthesis reaction of nitric oxide.

### Nitric oxide synthase

While NO is one of the smallest and simplest biological molecules, the NOS molecules are among the largest (~300 kDa) and most complicated. NOS exists in at least three different isoforms (Hecker, Walsh et al. 1991); one calcium-independent inducible NOS (iNOS, NOS II), and two calcium-dependent constitutive NOS (cNOS). The two forms of cNOS are named after the cell types in which they first were characterised; endothelial NOS (eNOS, NOS III) (Palmer and Moncada 1989; Marsden, Schappert et al. 1992), and neural NOS (nNOS, NOS I) (Nakane, Schmidt et al. 1993). However, these isoforms have later been found in skeletal muscle, endometrium, neutrophils, pancreatic islets, and in respiratory and GI epithelia. eNOS first purified and cloned in endothelial cells is also expressed in neurons (Dinerman, Dawson et al. 1994) and iNOS first purified and cloned in macrophages is also inducible in other cell types, among

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them neurons and endothelial cells (Oswald, Eltoun et al. 1994). Because of the confusing nomenclatures nNOS, iNOS, and eNOS the simplified numerical nomenclature (I, II, and III, respectively) defined by Schmidt et al. is also being used. (Schmidt, Pollock et al. 1991).

iNOS is absent in resting cells and more often expression of iNOS is reserved for infection or inflammation and geared toward host defence. The expression is controlled by the transcription factor nuclear factor (NF)- $\kappa$ B, which is activated by bacterial lipopolysaccharides (LPS) and pro-inflammatory cytokines such as TNF- $\alpha$ , interleukin (IL)-1 $\beta$  and interferon (INF)- $\gamma$  (Xie, Kashiwabara et al. 1994; Kolios, Rooney et al. 1998).

When iNOS is activated it synthesises NO in high (micromolar) amounts compared to the production of NO by cNOS, which is regulated by agonists (e.g. acetylcholine (ACh) and glutamate) or physical events such as shear stress resulting in intracellular calcium concentrations, generating NO at a low level (pico- to nanomolar) amounts (Dijkstra, van Goor et al. 2004).

### **NO in the gastrointestinal tract**

NO is involved in many of the processes in the GI tract. To protect the body from invading microorganisms the GI mucosa forms a barrier. NO has an important role in maintaining the integrity of this barrier which is composed of a layer of intestinal epithelial cells, mucus, acid, and non-specific agents such as lysozyme. NO is also involved in the regulation of intestinal peristalsis and gastric emptying (Orihata and Sarna 1996).

### **NO in inflammation and IBD**

In 1986 Roediger et al. (Roediger, Lawson et al. 1986) reported high levels of NO<sub>2</sub><sup>-</sup> in rectal dialysates from patients with UC and in the early 1990's various studies involving both animal models and humans indicated that NO may be involved in GI inflammation (Middleton, Shorthouse et al. 1993; Lundberg, Hellström et al. 1994). Whether NO acts primarily aggressively or protectively in IBD is still unclear, as NO can be protective as well as cytotoxic, having both anti- as well as pro-inflammatory properties (Pavlick, Laroux et al. 2002). When NO reacts with superoxide anions, peroxynitrate is formed which is a strongly toxic oxidant with several reaction pathways such as inhibiting enzymes involved in mitochondrial respiration and damaging DNA directly. Under normal conditions superoxide dismutase reacts with superoxide anions preventing the formation of peroxynitrate but in inflammatory conditions in which NO production is increased, peroxynitrate formation may dominate (Crow and Beckman 1995; Dawson 1995). Thus, in addition to acting as a signalling molecule, NO also exerts direct or indirect cytotoxic effects.

## Leukocyte recruitment

The microcirculatory changes that characterise inflammation may be caused by factors of physical, chemical, biological or immunological nature (Granger, Schelling et al. 1988). Recruitment of circulating leukocytes to the intestinal mucosa and leukocyte-mediated tissue injury are important parts of the pathophysiology of IBD (Powrie 1995; Fiocchi 1998; van Assche and Rutgeerts 2002). Specific inflammatory mediators generated at sites of infection or tissue injury initiate the inflammatory response. Guided by chemotactic signals, leukocytes then escape from the vasculature and migrate through the extravascular space to reach sites of infection or tissue injury (Zigmond and Hirsch 1973; Springer 1994; Lindbom and Werr 2002). This is due to interactions between adhesion molecules expressed on the cell membrane and on the endothelium. There are several families of adhesion molecules and one of them are integrins (Larson and Springer 1990).

### Integrins

Integrins are a family of heterodimeric receptors that are composed of two non-covalently linked protein chains; one  $\alpha$  and one  $\beta$  chain which bind to cytoskeletal proteins (Hynes 1987). There are more than 20 different integrin receptors, made out of different types of  $\alpha$  chains associated with different  $\beta$  chains. The  $\beta 1$  integrin family comprises at least ten different receptors that specifically bind to extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin. Members of the  $\beta 1$  integrin receptor family are expressed in almost all tissue cells and has been shown to be up-regulated in leukocytes during emigration and extravascular migration, and appear to be critically involved in regulating the immune cell trafficking from blood to tissue (Hemler 1990; Carlos and Harlan 1994; Brakebusch, Hirsch et al. 1997; Werr, Xie et al. 1998).

The  $\alpha 2\beta 1$  integrin (CD49b/CD29) is a collagen receptor widely expressed on tissue cells, whereas expression on leukocytes is seen only on specific activation, such as inflammation (Werr, Johansson et al. 2000).

### Integrins as drug targets

Compounds directed against cell adhesion molecules have been tested as treatment for CD and UC and to date, several cell adhesion molecules have been demonstrated to regulate disease activity in experimental colitis (Kato, Hokari et al. 2000; Soriano, Salas et al. 2000; Krieglstein, Cerwinka et al. 2002; van Assche and Rutgeerts 2002; Feagan, Yan et al. 2003; von Andrian and Engelhardt 2003; Farkas, Hornung et al. 2005; Lundberg, Lindholm et al. 2006) and in human trials (Danese, Semeraro et al. 2005). Integrins are attractive drug targets as their antagonism can block several steps in disease progression or maintenance;

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integrin inhibitors can block the proliferation, migration, or tissue localisation of inflammatory cells as well as signalling and gene expression contributing to disease (Staunton, Lupper et al. 2006). Reports have documented a role for  $\alpha 1\beta 1$  and  $\alpha 4\beta 1$  integrin receptors in IBD and concluded that these receptors have an important regulatory function in the initiation and perpetuation of the disease (Kato, Hokari et al. 2000; Soriano, Salas et al. 2000; Krieglstein, Cerwinka et al. 2002).

## **Experimental colitis**

Animal models of disease are important tools for etiology studies and pre-clinical studies of drugs. There is an active search for alternative systems not using animals, but some parameters are still impossible to study without using experimental animals. There are several models for experimental colitis, including:

### **Dextran sodium sulfate**

The dextran sodium sulfate (DSS) model, originally reported by Okayasu et al. (Okayasu, Hatakeyama et al. 1990) where the acute or chronic colitis is induced by DSS in their drinking water, is commonly used to investigate the role of leukocytes in intestinal inflammation. Clinical features e.g. signs of diarrhoea, gross rectal bleeding, and weight loss as well as histological features e.g. multiple erosions, inflammatory changes including crypt abscesses, and shortening of the large intestine, of the DSS model resemble IBD in humans (Okayasu, Hatakeyama et al. 1990; Krieglstein, Cerwinka et al. 2002; Lundberg, Lindholm et al. 2006).

### **Lipopolysaccharide**

Endotoxins are isolated from the cell wall of gram-negative bacteria and consist of LPS (Heath, Mayer et al. 1966; Ulevitch 1993; Apicella, Griffiss et al. 1994). In man and animal, LPS has extensive systemic effects, such as fever, hypotension, inhibited gastric and intestinal motility, and metabolic abnormalities (Ulevitch 1993; Hellström, Al-Saffar et al. 1997). The LPS model that induces acute colitis was first used on rabbits (Hotta, Yoshida et al. 1986) and has since been developed for several species.

### **Trinitrobenzene sulfonic acid**

Administration of the hapten 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 50% ethanol as the barrier breaker of the gastric epithelium, produces colonic ulceration and inflammation (Morris, Beck et al. 1989). The TNBS-model has been developed for IBD studies in both rats and mice (Duchmann, Schmitt et al. 1996). A recent study shows that intrarectal administration of TNBS to rats

influences not only their colon and terminal ileum, but also the proximal ileum and jejunum. This increase the relation of the model to IBD, maybe due to the systemic reaction of the immune system and mucosa seen in IBD (Amit-Romach, Reifen et al. 2006).



## AIMS

To investigate which subtype of NOS is activated in IBD and in experimental colitis.

To explore the expression of iNOS as a biomarker for inflammation in both animal and man.

To explore rectal NO as a biomarker of treatment response in UC and CD.

To examine relationships between rectal NO and mucosal expression of NOS in IBD.

To investigate the role of the collagen-binding  $\alpha 2\beta 1$  integrin in experimental colitis.

To compare the therapeutic effect of anti-integrin treatment directed against  $\alpha 2$  (CD49b) and  $\alpha 4$  (CD49d), to betamethasone, methotrexate, 5-ASA, and azathioprine in experimental colitis.



## MATERIAL AND METHODS

### Experimental colitis

#### Animals

The studies were approved by the Regional Ethics Committee for the Humane use of Research Animals in Northern Stockholm. All animals were kept under standardised conditions of temperature (21°C) and illumination (12:12-h light/darkness) at the Animal department at Karolinska Hospital, Stockholm, Sweden. Food and drinking water were available *ad libitum*.

#### Lipopolysaccharide-induced colitis

In paper I, acute colitis was induced in Sprague-Dawley rats (Scanbur BK, Sollentuna, Sweden) by i.v. administration of LPS from *E. coli* 0111.B4 (Sigma, St Louis, MO, US) at a dose of 100 µg kg<sup>-1</sup>. Healthy control rats were given saline i.v. The animals were killed with an overdose of sodium pentobarbital (Apoteket AB, Stockholm, Sweden) injected intra-peritoneally 90 min after administration of LPS or saline, and tissue sample for studies on gastrointestinal motility and for determination of NOS gene expression was collected.

#### Studies on gastrointestinal motility *in vitro*

In the *in vitro* studies, 12 mm muscle strips of the rat colon were mounted (Fig. 3) with a counter weight of 1 g (9.81 mN) in 5 ml organ bath chambers (AD Instruments,

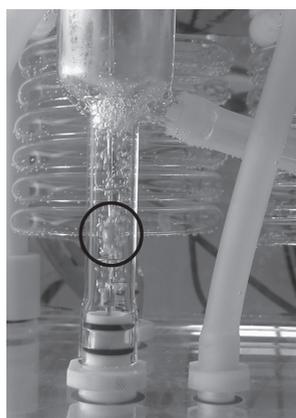


Figure 3. Rat colon (in the black ring) mounted in a 5 ml organ bath chambers containing continuously oxygenated modified Krebs-Ringer buffer.

Oxford, UK) containing continuously oxygenated (5% CO<sub>2</sub>, 95% O<sub>2</sub>) modified Krebs-Ringer buffer. The temperature was maintained at 37°C. The lower end of the tissue segments was anchored to the bottom of the chamber and the other end connected to a transducer. Isometric contractions were continuously recorded with Chart 4.1™ Software (AD Instruments, Oxford, UK). After equilibration, contractile effects of ACh (Sigma-Aldrich, Steinheim, Germany) were evaluated on both inflamed and normal colon, at concentrations of 10<sup>-8</sup>- 10<sup>-3</sup> M.

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The effect of the NOS inhibitor N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) (Sigma, St Louis, MO, US) on contractility of inflamed muscle strips was studied. The strips were incubated with L-NAME for 10 min before challenge with ACh. Glyceryl trinitrate used as a NO-donor was added to healthy contracted muscle strips in order to validate the effect of NO on colonic muscle.

In separate control experiments the effects of ACh and electric field stimulation (EFS) on the contractility of colonic muscle were studied in both the absence and presence of tetrodotoxin (TTX) (Sigma-Aldrich, Stockholm, Sweden). TTX is a potent neurotoxin, which blocks action potentials along nerve fibres and axons by binding to the pores of the voltage-gated sodium channels in nerve cell membranes. The binding site of this toxin is located at the pore opening of the voltage-gated Na<sup>+</sup>-channel.

### **Expression of nitric oxide synthase**

The expression of eNOS, nNOS and iNOS was determined in rat colonic tissue and the expression of iNOS in mice colonic tissue, with reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was isolated using RNeasy mini kit and RNase-free DNase set (Qiagen, Hilden, Germany). One µg of total RNA from each preparation was used to synthesise single-stranded cDNA. The obtained cDNA served as a template for the PCR. This resulted in cDNA fragments 210 bp for eNOS, 210 bp for nNOS, and 170 bp for iNOS. PCR fragments were loaded on a 2% agarose gel. After electrophoresis DNA, bands were visualised with ethidium bromide under UV light, and confirmed against positive and negative controls.

### **Dextran sodium sulfate-induced colitis**

The experiments (paper III and IV) in DSS-induced colitis were conducted in seven week-old (paper III) and in nine week-old (paper IV) female BALB/c mice (Scanbur BK, Sollentuna, Sweden). Colitis was induced by 2.0% (paper III) and 2.5% (paper IV) DSS (mol. Wt 40kD; TdB Consultancy, Uppsala, Sweden), dissolved in purified drinking water, for 12 days (paper III) and for 19 days (paper IV). In both experiments the treatment groups were compared with untreated control mice that received purified drinking water. In paper III, the treatment started at the same time as the DSS was given. In paper IV, the treatment started first after onset of clinical signs of colitis, which enabled us to monitor potential degree of clinical remission. All treatment groups in paper IV received daily active compound or vehicle, from day 13 until the end of the experiment, day 19.

### Antibodies and drugs

In paper III, the mice given DSS were divided into three groups; one group received function-blocking monoclonal antibody (mAb) Ha 1/29 against the  $\alpha 2$  ubunit (CD49b), one group received the isotype mAb Ha 4/8 (both hamster anti-rat mAbs from Pharmingen, San Diego, CA, US), and the third group received betamethasone (Apoteket AB, Stockholm, Sweden), in their drinking water. In paper IV, the mice given DSS were divided in five groups. One group received AZA (Imurel<sup>®</sup>, Apoteket AB, Stockholm, Sweden) in their drinking water. The other mice received daily rectal treatment with either methotrexate (MediGelium AB, Stockholm, Sweden), mesalazine (Pentasa<sup>®</sup>, Apoteket AB, Stockholm, Sweden), anti-integrin mAb directed against  $\alpha 2$  (CD49b) of the  $\alpha 2\beta 1$  integrin (CD49b/CD29) or  $\alpha 4$  (CD49d) of the  $\alpha 4\beta 1$  integrin (CD49d/CD29). In paper III, antibodies were administrated in a dose of 20  $\mu$ g in 80  $\mu$ L purified water and in paper IV, the amount of water was decreased to 50  $\mu$ L to maximise the antibody uptake in the colon. In both paper III and IV, antibodies and drugs that were administrated rectally were administered approximately four cm above the anal sphincter by using a small catheter, a shortened X-Ray Opaque (XRO) feeding tube (Vygon, Ecouen, France) originally used for premature babies (Fig. 4).

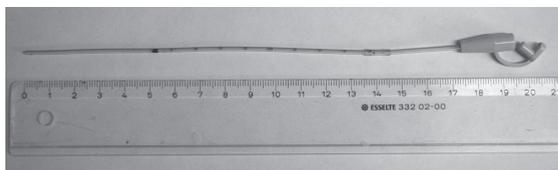


Figure 4. The catheter, a shortened X-Ray Opaque feeding tube, employed for rectal administration of drugs to mice.

### Clinical assessment of colitis

Daily assessment of all mice included measurement of body weight, drinking water volume and evaluation of blood in faeces and diarrhoea. Faecal blood was analysed with Hemocult IVD (TRIOLAB, Mölndal, Sweden).

### Histopathological assessment of colitis

All of the animals were killed at day 14 (paper III) and at day 19 (paper IV), and their colon was removed. Colon lengths were measured in fresh specimens. Samples for RNA extraction to microarray analysis and for determination of NOS gene expression were obtained from the distal colon (paper III). The freshly obtained colon was then fixated in 4% formaldehyde and embedded in paraffin before staining with haematoxylin-eosin. Histological quantification of

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mucosal damage and inflammation was performed along the entire longitudinal sections of the specimens in paper III and in the most distal part in paper IV. Specimens and treatment groups were blinded before histological quantification. Histological scoring was performed by the use of two independent parameters: percentage of mucosal surface affected by lesions and neutrophil count in mucosal lesions. Lesions were defined as visible damage of the mucosal surface involving more than two thirds of the total mucosal thickness. Cell count of haematoxylin-eosin-stained neutrophils was consistently performed in three high-power fields (40 x lens covering 0.45 x 0.45 mm) in the lamina propria in the most distal lesion in each animal. Neutrophils were identified through high magnification of haematoxylin-stained nuclei. The inflammatory response in paper IV was graded by morphological microscopic analysis of the colonic mucosa by an experienced GI pathologist (JL). A subjective grading system of acute inflammatory activity with four grades was adopted. Normal mucosa was graded as 0, sporadic scattered segmented granulocytes in the lamina propria was defined as grade 1, few granulocytes in smaller foci was defined as grade 2. Larger quantities of granulocytes in lamina propria or if granulocytes were seen in crypts, the inflammation was defined as grade 3. The surface extension of the mucosal damage was also quantified and expressed as percentage of total colon (paper III) or circular section of the distal colon (paper IV).

### **Gene array**

The GEArray Q Series mouse nitric oxide gene array from SuperArray (Bioscience, Frederick, MD, US) was used in paper III. Total RNA was isolated from the four groups; healthy control mice, DSS alone, anti- $\alpha 2$  mAb-treated, and betamethasone-treated mice. RNA from each group was used to generate  $^{32}\text{P}$  labeled cDNA probes. The cDNA probes were denatured and hybridised at 60°C with SuperArray membranes, which then were washed and exposed with the phosphor imager screens. The phosphor imager screens were scanned by a Fujifilm BAS 2500 machine and analysed with SuperArray's GEArray Expression Analysis Suite program (Bioscience, Frederick, MD, US). The average signal intensities of two glycerol aldehyde phosphate dehydrogenase (GAPDH) and two  $\beta$  actin spots were used as positive controls and set as baseline values with which the signal intensity of other spots were compared. Using this normalised data, the signal intensity from the membranes was compared.

## Studies in patients with IBD

### Including criterias and registration of disease activity

The study (paper II) was approved by the Karolinska Institutet Ethics Committee North.

IBD patients treated with GCS due to active UC or CD were eligible to enter the study. Diagnosed by endoscopic, radiological and histological criteria of IBD (Lennard-Jones, Lockhart-Mummery et al. 1968), 22 patients with UC and 24 patients with CD were recruited to the study. The patients were studied at four different occasions during the first month of treatment; before onset of prednisolone treatment (day 1), and at three follow-up visits during ongoing treatment (day 3, 7, and 28). The control for immunohistochemistry analysis consisted of six individuals undergoing colonoscopy for polyp control. The control for rectal NO consisted of 25 healthy volunteers.

Disease activity was assessed using the DAI for UC and HBI for CD. The endoscopic classification was done according to DAI for all patients. At the last visit (day 28) the patients were divided into responders (remission) versus nonresponders (no remission) for subgroup analysis. Remission was defined as  $DAI \leq 2$  in UC, and  $HBI \leq 4$  was used to define remission in CD, whereas nonresponders were defined as  $DAI \geq 3$  and  $HBI \geq 5$  in UC and CD, respectively.

### Sampling and determination of rectal NO

The fact that NO is stable at low concentrations in ambient air, diffuses easily, and in the colon is spread within the extension of the lumen provides the basis for



Figure 5. The balloon catheter, connected to a syringe, employed for sampling of rectal nitric oxide. Printed with permission from Dr Claudia Reinders.

rectal sampling with a balloon catheter (Body, Hartigan et al. 1995; Herulf, Ljung et al. 1998). The catheter (Fig. 5) (Argyle™, Sherwood Medical, Tullamore, Ireland) is made entirely of silicone to minimize allergic reactions.

The catheter was inserted into the rectum to a level of 10 cm above the anal sphincter. The balloon was then inflated with 10 ml of ambient air

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(<5 parts per billion (ppb) NO) and then left for 10 min to equilibrate with gases in rectum. The gas sample was subsequently withdrawn from the catheter balloon and diluted to a final volume of 50 ml for chemiluminescence analysis of NO (CLD 700, Eco Physics, Dürnten, Switzerland). The analyser was calibrated at known concentrations (100-10,000 ppb) of NO in nitrogen gas (AGA, Lidingö, Sweden). The chemiluminescence assay is highly specific for NO without interference from other nitrogen oxides (Archer 1993).

## RESULT AND COMMENTS

### Contraction studies

Control studies with muscle strips from normal rats displayed a dose–response relationship for ACh ranging from  $10^{-8}$  to  $10^{-3}$  M (all  $p < 0.05$ ). Inflamed colonic tissue showed a reduced contractile responsiveness, thus the  $pD_2$ -value decreased from  $7.09 \pm 0.17$  to  $5.30 \pm 0.19$  ( $p < 0.001$ ) (Fig. 6), and decreased contraction efficacy to  $78 \pm 5\%$  ( $p = 0.047$ ) of the maximal response to ACh in healthy controls. Inhibition of NO production by L-NAME reversed the  $pD_2$ -value from  $5.30 \pm 0.17$  to  $6.60 \pm 0.19$  ( $p < 0.001$ ) and the contractile efficacy to  $96 \pm 5\%$  (ns compared to control efficacy). L-NAME did not change contraction efficacy when

added to control colonic tissue strips ( $pD_2 = 6.12 \pm 0.37$  ns).

Glyceryl trinitrate decreased the contractile response to ACh seen in colonic tissue strips ( $p < 0.05$ ). In additional control experiments, the contractile effect of EFS on colonic tissue strips was abolished by TTX, whereas a comparable contractile response to ACh was not ( $p < 0.05$ ) which shows the experimental system to be independent of neuronal tissue.

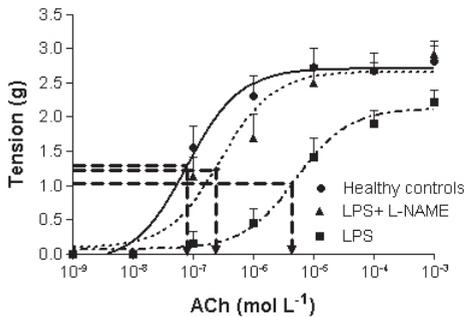
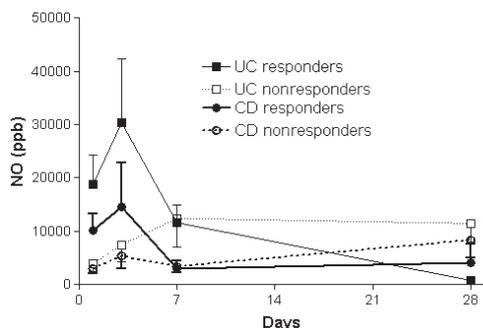


Figure 6. Dose-response relationship and  $pD_2$ -values for the contractile response in colonic rat tissue to acetylcholine (ACh) alone or in combination with  $N^{\omega}$ -nitro-L-arginine methyl ester (L-NAME) in healthy control rats and in rats with lipopolysaccharide (LPS)-induced colitis

### Rectal NO levels

On day 1, patients with active UC and CD had greatly increased NO levels ( $10,950 \pm 7,610$  and  $5,040 \pm 1,280$  ppb, respectively) as compared with the controls ( $154 \pm 71$  ppb, all  $p < 0.001$ ). Rectal NO showed a numerical increase on day 3 compared to day 1, after which a decrease was seen (Fig. 7). This pattern was mainly attributed to the patients responding to GCS treatment. Nonresponders displayed a less prominent increase on day 3, and showed no subsequent decrease. In both UC and CD patients that responded to GCS treatment, rectal NO levels decreased significantly between day 1 and day 28 (from  $18,860 \pm 5,390$  to  $850 \pm 450$  ppb in UC,  $p < 0.001$  and from  $10,060 \pm 3,200$  to  $4,130 \pm 3,380$  ppb in CD,  $p < 0.05$ ).

Figure 7. Rectal NO levels in ulcerative colitis and Crohn's disease patients subdivided into responders (disease activity index (DAI)  $\leq 3$  and Harvey Bradshaw index (HBI)  $\leq 5$ ), and nonresponders (DAI  $\geq 3$  and HBI  $\geq 5$ ) on day 28.



A different rectal NO pattern was seen among the patients in whom colectomy was carried out. Their rectal NO levels were significantly lower at day 1 ( $620 \pm 270$  ppb in UC, and  $1260 \pm 550$  ppb in CD) compared to corresponding levels in the patients with a treatment response ( $p < 0.001$  for UC and  $p < 0.05$  for CD).

## Expression of nitric oxide synthase

Using primers specific for eNOS, nNOS and iNOS in rat (paper I), distinct RT-PCR products of predicted sizes; 210 bp, 210 bp and 170 bp, respectively were obtained from rats treated with endotoxin. In control rats, distinct RT-PCR products were found for eNOS and nNOS-specific primers. In mice with experimental colitis, a distinct RT-PCR product was found for iNOS. This product was not found in control mice (thesis). The iNOS expression was regularly not observed in healthy controls, even if a few cases disclosed a faint band in the same position as iNOS. This observation of a weak expression of iNOS also in some controls indicates that other factors than endotoxin may be responsible for an activation of iNOS. One such factor may be the stressful events or merely handling of the tissue, which may evoke some activation of iNOS expression (Colon, Madrigal et al. 2004).

As shown by immunohistochemistry, the number of iNOS expressing cells was significantly higher in patients with UC and CD on day 1 compared with the healthy controls (all  $p < 0.001$ ). Semi-quantitative RT-PCR also showed significantly over-expression of iNOS in inflamed mucosa compared to normal parts of the mucosa ( $p < 0.05$ ) (paper II), which confirms our data from colitis in mice and rats.

Similar as the iNOS expression, TNF- $\alpha$  and IL-1 $\beta$  expressions were down-regulated in response to GCS treatment in IBD-patients with a decline of cytokine expression most pronounced at the follow-up visit on day 28 (paper II).

## Histopathology

In rats (paper I) subjected to LPS, a clear-cut inflammatory response was evident as shown by invasion of white blood cells, mainly neutrophils, oedema, and tissue disintegration.

In mice with DSS-induced colitis (paper III and IV), histological examination revealed mucosal lesions, oedema, crypt damage, and inflammatory infiltrates. Methotrexate, AZA, and anti- $\alpha$ 2 integrin could all ameliorate both severity and extension of mucosal damage and in all three treatment groups some of the animals had an undamaged mucosa, most animals with fully intact mucosa were found in the anti- $\alpha$ 2-treated group.

### **Effect of anti- $\alpha$ 2 monoclonal antibodies on mucosal lesions**

The DSS-induced colitis was characterised by mucosal lesions in the distal colon. In paper III, longitudinal sections of the distal colon were analysed for mucosal lesions. The lengths of all lesions in one section were added and divided by the total length of the specimen to obtain the percentage of mucosa affected by lesions. In mice receiving DSS alone,  $37 \pm 11\%$  of the mucosa analysed in a longitudinal section of the distal colon, were affected by lesions. In mice treated with anti- $\alpha$ 2 mAb,  $7 \pm 2\%$  of the mucosa were affected by lesions ( $p = 0.015$  vs. DSS alone). Betamethasone also protected against mucosal lesions ( $16 \pm 4\%$ ), although to a lesser extent than anti- $\alpha$ 2 mAb treatment. In paper IV, the most distal part was analysed. In mice only receiving DSS,  $57 \pm 13\%$  of the mucosa in the circular section were affected by lesions. In mice treated with anti- $\alpha$ 2 integrin mAb  $31 \pm 17\%$  of the mucosa in the circular section were affected.

### **Effect of anti- $\alpha$ 2 monoclonal antibodies on neutrophil recruitment**

The prevalence of neutrophils in mucosal lesions in the distal colon was assessed in paper III by cell count of neutrophils in a defined area within lesions. Anti- $\alpha$ 2 mAb treatment resulted in a dramatic reduction of neutrophil presence in mucosal lesions, from  $47 \pm 10$  neutrophils (per three high-power fields) in animals receiving only DSS to  $7 \pm 8$  neutrophils in the group receiving anti- $\alpha$ 2 mAb as well ( $p = 0.007$ ). Betamethasone resulted in reduction of neutrophil influx ( $30 \pm 12$  neutrophils;  $p = \text{NS}$  for trend), but not at the same magnitude as seen after anti- $\alpha$ 2 treatment. To further clarify the effect of anti- $\alpha$ 2 mAb treatment on neutrophil recruitment, comparative analysis of the distribution of neutrophils within colonic lesions were performed. As shown in the representative images in paper III and IV, transmural infiltration of neutrophils was seen in animals receiving just DSS. In contrast, in anti- $\alpha$ 2-treated animals, neutrophil infiltration was less pronounced (as quantified in the cell count) and located predominantly to the mucosa.

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## General signs of colitis

All mice that received regular or purified drinking water were negative for general signs of colitis throughout all the experiments.

Animals treated with anti- $\alpha 2$  mAb preserved their activity level and their fur-cleaning ability, whereas animal treated with no active compound lost their activity level and cleaning behaviour during the latter phase of the induced disease in all experiments.

### Body weight

In the same way as patients with IBD loose weight, animals that received DSS lost body weight during all experiments;  $14 \pm 7\%$  (paper III) and  $11 \pm 1\%$  (paper IV) lower body weight than healthy controls. In paper III, rectal administration of anti- $\alpha 2$  mAb was found to significantly reduce weight loss from  $14 \pm 7\%$  to  $2 \pm 0.2\%$  ( $p = 0.013$ ) while only a trend of reduced weight loss were seen in paper IV, where the treatment were started first after signs of colitis had occurred. In the latter study, methotrexate was the only treatment that prevented weight loss ( $3 \pm 2\%$  decrease in weight vs. DSS alone,  $p < 0.05$ ).

### Diarrhoea and rectal bleeding

Among all treatments tested in paper IV, only integrin antibodies significantly reduced rectal bleeding and diarrhoea compared to animals only receiving DSS. In new unpublished data from our group, where mice had a mild form of colitis, rectal administration of  $\alpha 2$  mAb was the only treatment that could totally prevent both diarrhoea and rectal bleeding to that point that these animals were comparable to control mice. Rectal administration of  $\alpha 4$  mAb did only show a trend in reducing these parameters.

### Colon length

Colon length is an indicator of colitis in both animals and man (Axelsson 1996). Colon length in healthy mice was  $9.1 \pm 1.0$  cm in paper III and  $9.5 \pm 0.4$  cm in paper IV. With DSS alone the colon length decreased to  $5.8 \pm 0.8$  cm and  $6.0 \pm 0.1$  cm (paper III and IV, respectively). In figure 8, a summary of colon lengths from both published and new unpublished studies are shown.

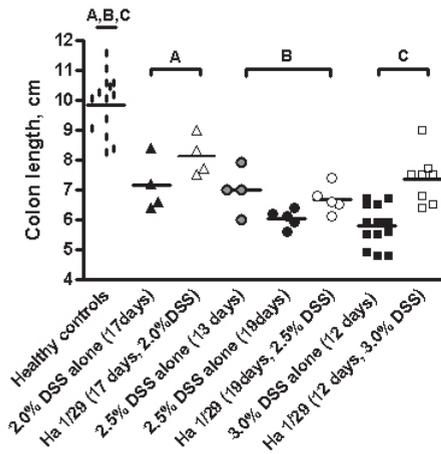


Figure 8. Colon length in healthy mice and in mice induced with different concentration and duration of DSS and in mice treated with Ha 1/29 (anti- $\alpha 2\beta 1$  integrin) in respectively study. A: unpublished data, Ha 1/29 was given day 2-7 from start of DSS. B: paper IV. C: paper III.

## Metalloproteinases

### Effect of anti- $\alpha 2$ monoclonal antibody on metalloproteinases

Expressions of metalloproteinase (MMP) genes were analysed with GEArray (paper III). DSS-treatment resulted in significantly increased gene expression of MMP-2, -7, and -9 compared with healthy mice. Administration of anti- $\alpha 2$  mAb resulted in a complete down-regulation of all three MMPs to levels below those seen in healthy mice. The effect was most marked for MMP-7, with a 2.4 fold up-regulation in DSS-treated mice and complete suppression below detectable levels after anti- $\alpha 2$  mAb treatment. A similar pattern of down-regulated MMPs was seen after treatment with betamethasone.



## GENERAL DISCUSSION

### NO in IBD and experimental colitis

iNOS was shown to be the isoform of NOS accountable for the elaboration of NO in the GI tract during inflammation, both in experimental colitis and in active UC and CD. There are different opinions whether cNOS contribute to the increased NO production in IBD or not. In this thesis, no differences were detected between healthy controls, active IBD, and IBD in remission, when staining for eNOS and nNOS. Neither were any differences detected in gene expression of eNOS and nNOS in experimental colitis compared to healthy animals. The results show that expression of iNOS could be the biomarker for colonic inflammation, that today is missing.

The iNOS expression in polymorphonuclear leucocytes in the lamina propria showed significant correlation with rectal NO levels in IBD. This is in line with earlier studies indicating the cellular source of NO in colitis to be inflammatory cells, predominantly macrophages, neutrophils and eosinophils (Amin, Attur et al. 1995), and smooth muscle (Mourelle, Casellas et al. 1995).

NO is deemed to be important for the blunted contractile response to ACh in inflamed colon. In inflamed tissue, the contractile response to ACh was significantly reduced. This inhibition was reversed by addition of a NOS inhibitor, indicating involvement of NO in the suppression of smooth muscle contraction in inflamed tissue. In additional control experiments, the NO-donor glyceryl trinitrate inhibited contractions in muscle strips precontracted with ACh, confirming the expected inhibitory action of NO on the gut motility. Experiments with ACh and EFS in conjunction with TTX showed the contractile response to ACh to be independent of neuronal tissue, i.e. after TTX incubation the contractile response to ACh persisted, while a similar contractile response to EFS disappeared.

NO is released downstream the inflammatory cascade in colitis (Moncada, Palmer et al. 1991) and whether it acts primarily aggressively or protectively in IBD is debated. NO has pro-inflammatory properties by stimulating chemotaxis of neutrophils and monocytes (Belenky, Robbins et al. 1993; Belenky, Robbins et al. 1993), enhancing the production of cytokines (Lander, Sehajpal et al. 1993), and generating superoxide ions (Pou, Keaton et al. 1999). NO is acting protectively in its capability to exert anti-inflammatory actions by down-regulating leukocyte-endothelial cell adhesion (Kubes, Kurose et al. 1994), decrease microvascular permeability (Kubes, Reinhardt et al. 1995), decrease aggregation of platelets (Moncada, Palmer et al. 1991), and down-regulate NF- $\kappa$ B (Peng, Libby et al. 1995).

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It seems that a high amount of NO is toxic, but low levels of NO might be even worse for IBD patients. Thus, we observed that high rectal NO levels in the beginning of a flare is associated with a favourable clinical outcome consistent with the idea that NO may act as an endogenous inhibitor of an aggregated immune response (Pfeiffer and Qiu 1995; McCafferty, Mudgett et al. 1997). In this way rectal NO is a candidate to become a biomarker of treatment response in UC and CD. Induction of iNOS seems to be critical as a protective response to injury in intestinal inflammation, possibly by reducing leukocyte infiltration. Another possibility is that NO is an innocent bystander in the inflammatory process, i.e. the molecule itself does not actively participate in the inflammatory process but may just as well be a reliable biomarker of the disease process. Previously published data speak in favour of rectal NO as a reliable diagnostic tool in the clinical setting and as a separator between IBD and irritable bowel syndrome (Reinders, Herulf et al. 2005).

## **Experimental colitis**

It is important to have a reliable and reproducible model of IBD to be able to study and improve treatments for patient with these diseases. The DSS model is commonly used, in particular to investigate the role of leukocytes in intestinal inflammation. Clinical as well as histological features of the DSS model resemble human IBD. The immunopathogenesis of the model involves infiltration of neutrophils, monocytes and lymphocytes that resembles a chronic disease (Lundberg, Lindholm et al. 2006; Abdelbaqi, Chidlow et al. 2006). We have studied several parameters for IBD and by optimising the DSS model we have established a colitis model appropriate for further studies including new treatments of IBD. This experimental model is also appropriate for further studies on the role of NO in IBD as results in this thesis have shown that the regulation of gene expression and synthesis of NO, are similar to that in humans.

The methods to induce colitis with DSS differ between laboratories. Some researchers administer DSS in periods of five to seven days with periods without DSS in between, this to develop a chronic colitis. The way to induce colitis should be adjusted to the animal strain used. Our group have noticed the importance of performing pilot studies with every new batch of DSS as the potency can differ remarkably.

## The role of the collagen-binding $\alpha 2\beta 1$ integrin in experimental colitis

Leukocyte recruitment is a complex process regulated by adhesion molecule interactions between leukocytes and endothelial cells and ECM proteins (Downey 1994; Springer 1994). Recruitment of circulating leukocytes to the intestinal mucosa is a pivotal step in the initiation and perpetuation of IBD (van Assche and Rutgeerts 2002). Therapeutic compounds directed against specific cell adhesion molecules have been tested as a treatment for CD and UC, which have led to a growing body of evidence that several different cell adhesion molecules could function as specific targets for therapeutic interventions in IBD. In paper III, the functional role of the collagen-binding integrin receptor  $\alpha 2\beta 1$  in DSS colitis in mice was assessed. The expression of  $\alpha 2\beta 1$  in leukocytes was previously believed to be restricted to activated mononuclear leukocytes but later shown to be induced also in neutrophils on extravasation of these cells from the vasculature (Werr, Johansson et al. 2000; Ridger, Wagner et al. 2001). A critical role has been demonstrated for  $\alpha 2\beta 1$  integrin in neutrophil migration in extravascular tissue, suggesting a function of the receptor also in the pathogenesis of inflammatory disease (Werr, Johansson et al. 2000). In paper III, a critical role of  $\alpha 2\beta 1$  integrin in regulating DSS colitis is demonstrated. Daily rectal administration of a function-blocking antibody against  $\alpha 2\beta 1$  integrin could prevent weight loss and significantly reduce histopathological signs of disease in the colon of DSS-treated animals. We identified that disruption of  $\alpha 2\beta 1$  integrin-dependent adhesion leads to impaired neutrophil accumulation at sites of mucosal damage in the colon. Similarly, the extent of inflammatory changes in the colonic mucosa was significantly reduced through blockage of  $\alpha 2\beta 1$  integrin.

We also investigated whether blockade of  $\alpha 2\beta 1$  integrin affected the expression of MMPs, well known to be involved in the pathophysiology of IBD (Pallone and Monteleone 2001). MMPs are proteinases involved in the breakdown and remodelling of the ECM under a variety of physiological and pathological conditions. MMP-2 and MMP-9, collectively known as the gelatinases, are particularly important in the pathogenesis of IBD (von Lampe, Barthel et al. 2000; Medina, Videla et al. 2003; Naito, Takagi et al. 2004). Matrilysin, a MMP-7, has previously been associated with many different tumour types, and recent reports have shown important roles in inflammatory disorders (Wielockx, Libert et al. 2004). Our observation of an up-regulation of MMP gene expression in DSS colitis is in line with previous reports of elevated MMP expression in CD, UC, and several experimental models of colitis, including DSS colitis (Louis, Ribbens et al. 2000; von Lampe, Barthel et al. 2000; Pirila, Ramamurthy et al. 2003;

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Kirkegaard, Hansen et al. 2004). We observed up-regulation of MMP-2, -7, and -9 in DSS-treated animals. Anti- $\alpha 2\beta 1$  integrin treatment completely suppressed gene expression of all three MMPs below levels seen in healthy animals, indicating that the integrin receptor may play a direct or indirect regulatory function for MMP expression in the inflamed colonic tissue. Other studies have demonstrated a similarly important role for  $\alpha 1\beta 1$  integrin and monocytes in DSS colitis (Kato, Hokari et al. 2000; Krieglstein, Cerwinka et al. 2002). In these studies, it was shown that disruption of  $\alpha 1\beta 1$  affects both motility and cytokine production in monocytes. Both  $\alpha 1\beta 1$ - and  $\alpha 2\beta 1$  integrins bind to collagen with a preference to collagen type IV and collagen type I, respectively, and their matrix-binding capabilities are carefully regulated (Mendrick, Kelly et al. 1995). Interestingly, our data and the previous study by Krieglstein et al. (Krieglstein, Cerwinka et al. 2002) show that blockade of the collagen-binding integrins  $\alpha 1\beta 1$  or  $\alpha 2\beta 1$  independently of each other results in significantly reduced disease activity in DSS colitis. In addition, data obtained from clinical trials investigating the therapeutic effect of  $\alpha 4$  integrin blockage in IBD, demonstrate the involvement of additional integrin receptors in the pathogenesis of this disease (Gordon, Lai et al. 2001; Ghosh, Goldin et al. 2003). Thus, to date, a number of  $\beta 1$  integrins have been demonstrated to be involved in regulating IBD or experimental forms of IBD such as DSS colitis.

## Evaluation of different treatments in IBD

Anti-integrin treatment in IBD is emerging as a novel treatment strategy that most likely can be designed to become more effective than conventional treatment.

This thesis show that integrin blockade, both with antibodies against  $\alpha 2$  and  $\alpha 4$  integrin, were most effective in suppressing rectal bleeding and diarrhoea, whereas treatment with methotrexate was superior to anti-integrin treatment in preventing weight loss caused by DSS colitis. Anti- $\alpha 2$  treatment was more effective in preventing mucosal damage than anti- $\alpha 4$  treatment and the majority of animals in the anti- $\alpha 2$  treated group showed no mucosal damage what so ever. In line with clinical data on weight loss, methotrexate was most effective in reducing severity of inflammation. However, in contrast to anti- $\alpha 2$  treated animals, only one methotrexate-treated animal showed absence of mucosal damage. Thus, our data show different clinical and histological pattern of different treatment regimens. Most likely this is due to the different mechanism through which the administered compounds act on the DSS model. Whereas anti-integrin treatment is likely to regulate DSS colitis through direct modulation of leukocyte recruitment, both AZA and methotrexate intervene with the

inflammatory cascade at several levels. We believe that the capability to decrease rectal bleeding, diarrhoea and mucosal damage are favourable the methotrexate-effect on weight loss.

Compounds directed against cell adhesion molecules have been tested as treatment for CD and UC. To date, at least four different cell adhesion molecules have been demonstrated to regulate disease activity in experimental models of colitis (Kato, Hokari et al. 2000; Soriano, Salas et al. 2000; Krieglstein, Cerwinka et al. 2002; van Assche and Rutgeerts 2002; Feagan, Yan et al. 2003; von Andrian and Engelhardt 2003; Farkas, Hornung et al. 2005).

Observations in this thesis both support the concept that integrins, and in particular  $\beta 1$  integrins, play a major role in both intravascular and extravascular events of leukocyte recruitment in IBD, and show that anti-integrin regimens have a therapeutic potential beyond current treatment regimens with 5-ASA, betamethasone, immunomodulators, and cytostatic compounds. As treatment with antibodies against integrin  $\alpha 2\beta 1$  provides anti-inflammatory effect in experimental colitis, this may form the basis for a novel therapeutic concept in inflammatory bowel disease.

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## SUMMARY AND CONCLUSIONS

Active UC and CD are associated with highly increased rectal NO levels and increased expression of iNOS compared to healthy persons, which is also seen in experimental colitis in mice and rats.

Studies in experimental colitis as well as IBD in man open the possibility of NO as a biomarker of inflammatory disease activity in the gut.

Integrins, particularly  $\beta 1$  integrins, play a major role in both intravascular and extravascular events of leukocyte recruitment in IBD and may thus constitute targets for therapeutic interventions in IBD.

$\beta 1$  integrins have a therapeutic potential beyond current treatment regimens with 5-ASA, betamethasone, immunomodulators and cytostatic compounds, which are all associated with several side-effects.

The data demonstrate an alleviating action of the collagen binding  $\alpha 2\beta 1$  integrin in experimental colitis and suggest that this effect is mediated by inhibition of neutrophil migration and activation. Local administration of function-blocking antibodies against integrin  $\alpha 2\beta 1$  may provide novel avenues to treat IBD.

## SUMMARY IN SWEDISH

Ulcerös kolit (UC) och Crohns sjukdom (CD) är kroniskt inflammatoriska mag-tarmsjukdomar (IBD). Vid UC är inflammationen kontinuerlig från rektum och sträcker sig i varierande grad proximalt i kolon. Vid CD är inflammationen däremot diskontinuerlig och kan drabba hela mag-tarmkanalen, från munhålan till rektum. I Sverige är den totala prevalensen av IBD ca 1 %. Vanliga symptom associerade till aktiv IBD är diarré ofta innehållande blod och slem, buksmärtor och viktnedgång.

Etiologin till sjukdomarna är fortfarande oklar och någon behandling som botar sjukdomarna finns inte idag. Den medicinska behandlingen syftar till att uppnå symptomfrihet och endoskopisk remission. Den medicinska behandlingen är till stor del lika för UC och CD och består främst av 5-aminosalicylsyra (5-ASA), glukokortikoider och immunmodulerande medel för båda sjukdomarna. Alla patienter svarar dock inte på dessa läkemedel vilket inte heller är att förvänta mot bakgrund av att etiologin inte är känd. Detta innebär att fortsatt sökande efter nya effektiva läkemedel är oerhört viktigt.

Karaktäristiskt för IBD är att kvävemonooxid (NO) frisätts som gas lokalt i hög koncentration i tarmen. I avhandlingen presenteras resultat från olika experiment som visar att NO kan användas som biomarkör för att identifiera inflammation både hos människa och hos djur. Dessa human- och djurstudier visar att det är iNOS (ett av tre NO-syntaser), som ökar vid IBD och bidrar till att höga halter av NO bildas i kolon. Hur NO påverkar kolon och hur halten av rektalt NO är associerad till hur patienter svarar på läkemedelsbehandling studeras också i avhandlingen. NO frisätts som ett svar på inflammation och resultaten visar att en initial höjning av NO i det akuta skedet av IBD är normalt och verkar vara positivt för sjukdomsförloppet om man kan minska halten genom medicinering. Höga rektala halter av NO under lång tid är toxiskt och minskar tarmens motilitet. Resultaten visar att låg rektal NO-halt i det akuta skedet indikerar att inflammationen inte kommer att svara på läkemedelsbehandling, vilket för patienten kan innebära kolektomi.

Karaktäristiskt för IBD är även ansamling av leukocyter i den drabbade gastrointestinala vävnaden. Migrationen av leukocyter är noga styrd av adhesionsmolekyler, däribland integriner. Tidigare studier antyder att  $\beta$ 1-integrin-receptorer har betydelse för den vävnadsskada och de symptom som är relaterade till IBD. I avhandlingen studeras effekten av en monoklonal antikropp riktad mot den kollagenbindande  $\alpha$ 2 $\beta$ 1-integrin (CD49b/CD29)-receptorn i en experimentell kolitmodell på mus. Målet var att även jämföra denna behandling

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med de konventionella läkemedel som används idag. Såväl kliniska som histologiska analyser i denna avhandling visar positiva resultat vid behandling med integrinantikroppar. Störst framgång ses när antikropparna sätts in i ett tidigt skede av sjukdomen. Djur som behandlas med integrinantikroppar behåller sitt normala aktiva beteende och förlorar mindre vikt jämfört med de djur som behandlats konventionellt. Behandlingen med integrinantikroppar har även resulterat i färre lesioner i tarmen samt mindre anhopning av leukocyter vid lesionerna sedan de väl uppkommit. När behandling sattes in först efter att djuren etablerat kolit visades att fler djur gick i histologisk remission efter behandling med den monoklonala antikroppen riktad mot  $\alpha 2\beta 1$ -integrinreceptorn än efter konventionell medicinsk behandling.

Sammanfattningsvis visar avhandlingen att koncentrationen av rektalt NO och genuttrycket av iNOS i kolonvävnad kan användas som biomarkörer dels diagnostiskt för kolit och dels för monitorering av sjukdomsaktiviteten vid läkemedelsbehandling. Även en ny behandlingsmetod i form av en monoklonal antikropp riktad mot  $\alpha 2\beta 1$ -integrinreceptorn har utvärderats. De positiva resultaten på experimentell kolit kan innebära att detta är ett steg på vägen mot ny effektiv behandling mot kroniskt inflammatoriska mag-tarmsjukdomar.

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I



## Expression of iNOS mRNA associated with suppression of colonic contraction in rat colitis

S. Lundberg,<sup>1</sup> M. Holst<sup>2</sup> and P. M. Hellström<sup>1</sup>

<sup>1</sup> Department of Medicine, Unit of Gastroenterology and Hepatology, Karolinska University Hospital Solna, Karolinska Institutet, Stockholm, Sweden

<sup>2</sup> Department of Women and Child Health, Unit of Pediatric Endocrinology, Karolinska University Hospital Solna, Karolinska Institutet, Stockholm, Sweden

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Correspondence: S. Lundberg,  
Department of Gastroenterology  
and Hepatology, M1:02, Karolinska  
University Hospital Solna, SE-171  
76 Stockholm, Sweden.  
E-mail: Sofie.Lundberg@ki.se

### Abstract

**Aim:** Nitric oxide (NO) synthesis and inducible NO synthase (NOS) expression are increased in colon of patients with inflammatory bowel disease (IBD) and associated with decreased contractility. The aim was to investigate which subtype of NOS that is activated in experimental colitis.

**Methods:** Experimental colitis was induced in Sprague–Dawley rats by *Escherichia coli* endotoxin. Expression of different subtypes of NOS was compared in normal and inflamed colon using reverse transcriptase-polymerase chain reaction. In organ baths, isometric contractile responses to acetylcholine (ACh) were studied in the colon, before and after incubation with the NOS inhibitor; N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) and NO donor glyceryl trinitrate.

**Results:** Inflammation decreased colonic contraction to ACh from a pD<sub>2</sub> value of 7.09 ± 0.16 to 5.30 ± 0.17 (*P* < 0.001), and reduced maximal response to ACh. Pre-treatment with L-NAME reversed contractility and shifted the pD<sub>2</sub> for ACh from 5.30 ± 0.17 to 6.60 ± 0.19 (*P* < 0.001) along with a normalized contraction efficacy. RT-PCR product of iNOS was obtained only in rats treated with endotoxin.

**Conclusion:** Expression of iNOS is increased in inflamed colonic tissue. The induced overproduction of NO is likely to be responsible for the decreased motility in colitis where NO is suggested to exert a suppressive tone on colonic contractility, which is reversed by blockade of the enzyme.

**Keywords** gastrointestinal motility, inflammation, inflammatory bowel disease, nitric oxide, smooth muscle.

Inflammatory diseases of the colon are associated with motility disturbances, most frequently decreased contractility (Snape & Kao 1988, Reddy *et al.* 1991). Experimental colitis in the rat has been shown to decrease colon transit (Pons *et al.* 1994). In association with flare of inflammatory bowel disease (IBD), decreased contractility and distension of the gut are commonly encountered in the clinical setting (Mourelle *et al.* 1995, Rachmilewitz *et al.* 1995). The release of inflammatory mediators, such as prostanoids and plate-

let activating factor (PAF), interleukin-1, and leukotrienes in the afflicted tissue are assumed to take part in this effect (Lauritsen *et al.* 1986, Morneau *et al.* 1993, Pons *et al.* 1994).

Nitric oxide (NO) is involved in relaxation of gastrointestinal smooth muscle (Burleigh 1992, Pawlik *et al.* 1993), including the colon (Vannucchi *et al.* 2004). Overproduction of NO has been implicated in the pathogenesis of clinical and experimental IBD (Boughton-Smith *et al.* 1993a). The initiation of the

inflammatory cascade seems to involve both macrophages and inducible nitric oxide synthase (iNOS) and has been found to take place in macrophage-related cells in proximity to muscular elements (Zheng *et al.* 1997). Studies *in vitro* have shown endotoxin (lipopolysaccharide; LPS) to stimulate iNOS in gastric fundus (Takakura *et al.* 1996), as well as in macrophages of the rat jejunum, where it inhibits motor activity (Eskandari *et al.* 1999). *In vitro* studies of inflamed tissue from patients with IBD have shown decreased contractility (Al-Saffar & Hellström 2001). Recent studies by our group and others point at iNOS as the most likely source of the vast NO production in IBD (Dijkstra *et al.* 2002, Ljung *et al.* 2006, in press), as well as in experimental colitis (Colon *et al.* 2001). Furthermore, data show increased expression of iNOS in circulating monocytes from patients with active IBD (Dijkstra *et al.* 2002) pointing at a systemic involvement of the disease. However, in experimental colitis employing endothelial NOS-deficient (eNOS<sup>-/-</sup>) mice, a role has been ascribed to constitutive eNOS in maintaining the integrity of the mucosal lining and delimit tissue injury (Sasaki *et al.* 2003).

The aim of this interventional study was to induce colonic inflammation by the use of endotoxin and specifically determine which molecular subtype of NOS that is activated in this type of experimental colitis. In addition, colonic contractility was studied as a functional biomarker to determine the pathophysiological role of NO and its reversal by NOS inhibition under inflammatory conditions.

## Materials and methods

The regional ethics committee for the human use of research animals in northern Stockholm approved the study.

Experimental colitis was induced in 16 Sprague-Dawley rats by *i.v.* administration of LPS from *Escherichia coli* O111:B4 (Sigma, St Louis, MO, USA) at a dose of 100 µg kg<sup>-1</sup> and compared with 12 healthy controls. Tissue samples were collected for contractility studies and RNA isolation 90 min after administration of saline or LPS. Tissue samples were also taken for regular histology using 4% formalde-

hyde solution for fixation and haematoxylin-eosin for staining ( $n = 4$ ).

## Expression of nitric oxide synthase

The expression of eNOS, neuronal NOS (nNOS) and iNOS was determined in colonic tissue ( $n = 12$ ) with reverse transcriptase-polymerase chain reaction (RT-PCR). For each experiment, a parallel negative control without RT was processed and inflamed rat testis and kidney were used as positive controls for PCR under the same experimental conditions. First, total RNA was isolated using RNeasy mini kit and RNase-free DNase set (Qiagen, Hilden, Germany). About 1 µg of total RNA from each preparation was used to synthesize single-stranded cDNA using 200 units of RNase-H reverse transcriptase and oligodeoxynucleotide (25 µg mL<sup>-1</sup>; Invitrogen Ltd., Paisley, UK) as primer in a reaction volume of 20 µL. The obtained cDNA served as a template for the PCR, consisting of a denaturation step at 96 °C for 6 min, followed by 30 cycles of amplification (96 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min with a final extension duration of 5 min at 72 °C) using 2.5 U of DNA *taq* polymerase (Invitrogen) and specific primers for eNOS, iNOS and nNOS (Table 1). Primers were designed using Primer3 Output software and manufactured by CyberGene®, Stockholm, Sweden. This resulted in cDNA fragments 210 bp for eNOS, 210 bp for nNOS, and 170 bp for iNOS. PCR fragments were loaded on a 2% agarose gel. After electrophoresis DNA bands were visualized with ethidium bromide 0.5 µg mL<sup>-1</sup> under UV light and confirmed against positive and negative controls.

## Contraction studies

Muscle strips (3 × 12 mm) were cut along the longitudinal axis of the colon collected from inflamed ( $n = 8$ ) and normal ( $n = 10$ ) tissues. Strips were suspended in water-jacketed organ baths (AD Instruments, Oxford, UK) at 37 °C in 5 mL of Krebs solution containing (mmol L<sup>-1</sup>): NaCl 121, NaH<sub>2</sub>PO<sub>4</sub> 2.0, NaHCO<sub>3</sub> 15.5, KCl 5.9, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, D-glucose 11.5, oxygenated by 95% O<sub>2</sub>/5% CO<sub>2</sub> with a counter-weight of 1 g (9.81 mN). Isometric contractions were

**Table 1** Oligonucleotides of primers for PCR

Gene product	Sense	Antisense	Predicted size (bp)	Accession numbers
eNOS	5'-TGACCCTCACCGATAACA	5'-CTGGCCTTCTGCTCATITTC	210	NM021839
iNOS	5'-CACCTTGGAGTTCACCCA	5'-ACCACTCGTACTTGGGATGC	170	NM012611
nNOS	5'-CTGCAAAGCCCTAAGTCCAG	5'-AGCAGTGTTCCTCCTCCA	210	NM052799

eNOS, iNOS, nNOS are endothelial, inducible and neuronal nitric oxide synthase, respectively.

measured. Tissues were left to equilibrate for at least 60 min while the organ bath solution was changed regularly. After equilibration, contractile effects of acetylcholine (ACh) (Sigma) were evaluated at bath concentrations between  $10^{-9}$  and  $10^{-3}$  M administered as 50  $\mu$ L aliquots as single doses. The effect of the NOS inhibitor N<sup>o</sup>-nitro-L-arginine methyl ester (L-NAME)  $3 \times 10^{-4}$  mol L<sup>-1</sup> on contractility of inflamed muscle strips was studied by incubating the preparations for 10 min before challenge with ACh at the same concentration range. Glyceryl trinitrate used as a NO-donor was added to healthy contracted muscle strips at a concentration of  $4 \times 10^{-5}$  mol L<sup>-1</sup> in order to validate the effect of NO on colonic muscle. Glyceryl trinitrate was only added once to every muscle strip ( $n = 6$ ). In separate control experiments the effect of electric field stimulation (EFS) (50 V, 0.8 ms, 0.5–16 Hz) and ACh ( $10^{-8}$ – $10^{-4}$ ) mol L<sup>-1</sup> on the contractility of colonic muscle was studied in the absence or presence of tetrodotoxin (TTX)  $10^{-6}$  mol L<sup>-1</sup>.

#### Data analysis

Results of contraction studies were expressed relative to the maximal contraction obtained with ACh  $10^{-3}$  mol L<sup>-1</sup>, and values given as means  $\pm$  SEM. Concentration–response relationships and statistical evaluation were carried out with PRISM 3.0 (GraphPad, San Diego, CA, USA). Statistical significance was evaluated employing ANOVA or Student's *t*-test where appropriate.  $P < 0.05$  was considered statistically significant.

### Results

#### Histology

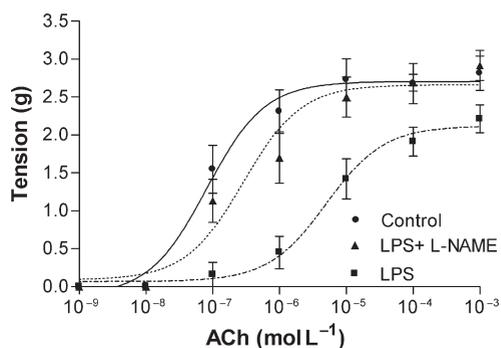
In all rats subjected to systemic administration of endotoxin a clear-cut inflammatory response was evident as shown by invasion of white blood cells, mainly neutrophils, oedema and tissue disintegration.

#### Expression of nitric oxide synthase

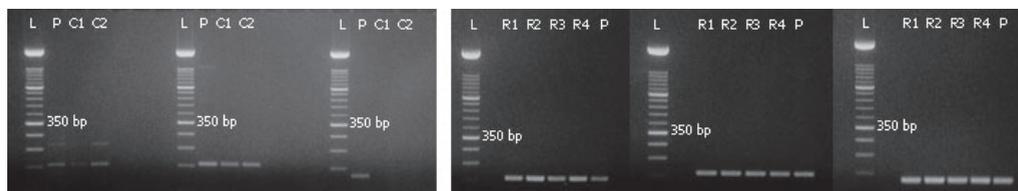
Using primers specific for eNOS, nNOS and iNOS distinct RT-PCR products of predicted sizes; 210 bp, 210 bp and 170 bp, respectively (Fig. 1) were obtained from rats treated with endotoxin. In control rats, distinct RT-PCR products were found for eNOS and nNOS-specific primers. The iNOS expression was regularly not observed, even if a few cases disclosed a faint band in the same position as iNOS. Parallel experiments without RT did not yield PCR products with any of the specific primers.

#### Contraction studies

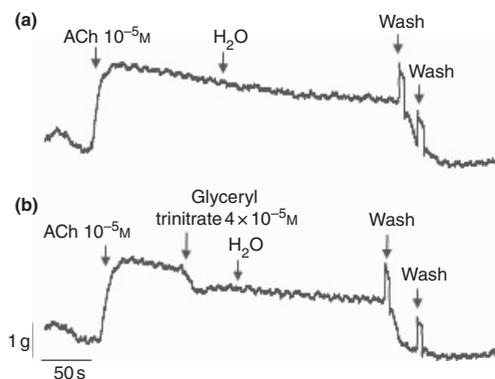
Control studies with muscle strips from normal rats displayed a dose–response relationship for ACh ranging from  $10^{-8}$  to  $10^{-3}$  mol L<sup>-1</sup> (all  $P < 0.05$ ), Figure 2. Inflamed colonic muscle showed a reduced contractile potency from a pD<sub>2</sub>-value of  $7.09 \pm 0.17$  to  $5.30 \pm 0.19$  ( $P < 0.001$ ), and a decreased contraction efficacy to  $78 \pm 5\%$  ( $P = 0.047$ , one-tailed test) of the maximal response to ACh. Inhibition of NO production by



**Figure 2** Dose–response relationship for the contractile response to acetylcholine (ACh) alone (control) or in combination with lipopolysaccharide (LPS) and LPS with N<sup>o</sup>-nitro-L-arginine methyl ester.



**Figure 1** Representative RT-PCR agarose gels showing the expression of inducible nitric oxide synthase (iNOS) in the colon of lipopolysaccharides (LPS)-treated rats. L denotes 50 bp PCR ladder, P positive control from inflamed rat kidney, C1 and C2 (left panel) denotes control experiments of eNOS, nNOS and iNOS, R1–R4 denotes a visual expression of eNOS, nNOS and iNOS in LPS-treated rats (right panel), of which eNOS and nNOS were expressed also under control conditions.



**Figure 3** Typical tracings showing the effect of glyceryl trinitrate ( $4 \times 10^{-5}$  M) on the contractile response from ACh ( $10^{-5}$  M) (a & b) on muscle strips from rat colon. As negative control 50  $\mu$ L  $H_2O$  was added (a & b).

L-NAME reversed the  $pD_2$ -value from  $5.30 \pm 0.17$  to  $6.60 \pm 0.19$  ( $P < 0.001$ ) and the contractile efficacy to  $96 \pm 5\%$  (ns compared to control efficacy). L-NAME did not change contraction efficacy when added to control colonic muscle strips ( $pD_2 = 6.12 \pm 0.37$ , ns). Glyceryl trinitrate decreased the contractile response to ACh seen in colonic muscle strips ( $P < 0.05$ ,  $n = 6$ ), Figure 3. In additional control experiments, the contractile effect of EFS on colonic muscle strips was abolished by TTX, whereas a comparable contractile response to ACh was not ( $P < 0.05$ ,  $n = 6$ ).

## Discussion

The aim of the study was to investigate the involvement of NO in acute experimental colitis in the rat. We found that NO is likely to be important for the blunted contractile response to ACh. The isoform of NOS responsible for the elaboration of NO was shown to be iNOS. In additional control experiments an NO-donor (glyceryl trinitrate) inhibited contractions in muscle strips precontracted with ACh, thereby disclosing the expected inhibitory action of NO on gut motility. Furthermore, experiments with ACh in conjunction with TTX showed the experimental system to be independent of neuronal tissue. Thus, after TTX the contractile response to ACh persisted, while a similar contractile response to EFS disappeared.

Our data confirm an inflammatory reaction in colonic tissue in response to systemic administration of endotoxin in a similar way as previously shown in the small bowel (Hellström *et al.* 1997). Furthermore, the present study showed a distinctly increased expression of iNOS mRNA in inflamed tissue in experimental colitis, while the expression of eNOS and nNOS were unchanged as

compared to control. The observation of a weak expression of iNOS also in some controls may indicate that other factors than endotoxin may be responsible for an activation of iNOS. One such factor may be the stressful events or merely handling of the tissue, which may evoke some activation of iNOS expression (Colon *et al.* 2004). In the inflamed tissue, the contractile response to ACh was greatly reduced. This inhibition was reversed by the addition of a NOS inhibitor, L-NAME, whereupon an immediate disinhibition was evident and the contractile response to ACh restored. Thus, data speak in favour of a key role for NO in the suppression of smooth muscle contraction in inflamed tissue.

Earlier work by Boughton-Smith and collaborators suggests that primarily iNOS is activated in intestinal injury (Boughton-Smith *et al.* 1993b). The vast production of NO through this enzyme is considered essential in inflammatory tissue reactions. As NO exerts similar smooth muscle-relaxing effects in intestinal smooth muscle and blood vessels (Umans & Levi 1995), this compound may be considered as a mediator of decreased smooth muscle tone and vasodilatation that are evident biomarkers of an inflammatory tissue response.

The cellular source of NO in colitis appears to be smooth muscle (Mourelle *et al.* 1995) and inflammatory cells, predominantly macrophages, neutrophils and eosinophils (Moncada *et al.* 1991, Amin *et al.* 1995, Del pozo *et al.* 1997, Wheeler *et al.* 1997, Cedergren *et al.* 2003). These cells are activated by bacterial endotoxins and cytokines such as tumor necrosis factor- $\alpha$ , interleukin- $1\beta$  and interferon- $\gamma$  (Bansal & Ochoa 2003), at which point iNOS produces vast amounts of NO limited only by substrate availability. Preliminary data from our laboratory indicate that these cytokines are expressed in colonic tissue from patients with ulcerative colitis and Crohn's disease, along with luminal NO overproduction (Ljung *et al.* 2006, in press).

The cause of smooth muscle relaxation in colitis is not known. A number of explanations for the inhibition of motility in inflammatory conditions have been forwarded. Among those, studies in IBD patients claim defective colonic muscle contraction (Snape *et al.* 1991, Cook *et al.* 2000, Al-Saffar & Hellström 2001), lowered luminal pressure (Reddy *et al.* 1991), and reduced post-prandial motility (Snape *et al.* 1980). Research suggests that these effects are due to changes in the tissue response to various neurotransmitters, most of all vasoactive intestinal peptide, substance P, neurotensin (Tomita *et al.* 2000), leukotrienes (Percy *et al.* 1990), and nitric oxide (Tomita & Tanjoh 1998). A recent study (Cao *et al.* 2004) demonstrates significantly elevated levels of the pro-inflammatory IL-1 in

the muscularis propria of patients with ulcerative colitis compared to controls concurrent with reduced smooth muscle contraction. The reduction in muscle fibre contraction was also reproduced in control tissue by addition of IL-1 at similar concentrations as found in diseased tissue. Furthermore, the restitution of contractile responses was achieved by the addition of catalase, a hydrogen peroxide scavenger, suggesting that hydrogen peroxide could be one of the mediators of inflammatory inhibition of motility (Cao *et al.* 2004). Hence, other free radicals, such as, e.g. NO may also be removed by the use of a scavenger possibly leading to disinhibition of inflammatory colonic relaxation.

Regardless of the primary step in colitis, downstream the inflammatory cascade NO is released (Moncada *et al.* 1991). NO is suggested to be the foremost non-adrenergic, non-cholinergic neurotransmitter in the gut and may be fundamental in inflammatory colonic relaxation. Employing enzyme kinetics Mourelle *et al.* (1995) demonstrated high NOS activity in colonic tissue from patients with toxic megacolon, whereas in uncomplicated colitis and tumour controls NOS activity was low or even undetectable. Extended studies in a rat colitis model using blockade of both Ca<sup>2+</sup>-dependent and -independent isoenzymes, made the increased NOS activity likely to be due to iNOS. As shown with trinitrobenzene sulphonate acid (TNBS) as colitis-inducing agent in another species (mice), iNOS is up-regulated with an increased expression (Vallance *et al.* 2004). This verifies a general importance of iNOS in colitis. Moreover, NOS activity was reduced by bowel decontamination using oral antibiotic pre-treatment (Mourelle *et al.* 1996) suggesting that bacteria and their products play a key role in the induction of NOS. By the use of a specific *E. coli* endotoxin to induce iNOS, both in earlier studies of the small bowel (Eskandari *et al.* 1999), as well as in the present study we have extended our data to imply that lipopolysaccharides have a fundamental role in activating iNOS in colitis. Whether NO is simply a marker of inflammation or truly involved in the pathogenesis of inflammatory tissue injury remains unclear.

In the present study, we found the colonic contractile responsiveness to ACh to be reduced about two orders of magnitude in inflamed tissue as compared to controls. The suppression of contractility was overcome by administration of a NOS inhibitor, which within a surprisingly short action time normalized the contractile responsiveness, both as regards potency and efficacy. An earlier study performed in rats with trinitrobenzene sulfonic acid induced colitis and NOS blockade restored the contractile response to KCl *in vitro* and increased basal intracolonic pressure in colitis rats *in vivo* (Mourelle *et al.* 1996). As the contractile response to KCl in colitis is blocked, it seems that the point of

inhibition is located within the cellular contractile elements. In addition, our present data show not only about a 100-fold reduction of the responsiveness to ACh, but also a reversible mechanism underlying inflammatory suppression of the contraction, as this could be overcome by increasing concentrations of the agonist. Thus, in inflammatory conditions NO elaboration as induced by iNOS from a multitude of cellular sources seems to be able to keep the tissue in a state of tonic relaxation via a reversible action on smooth muscle tissue.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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III





CLINICAL RESEARCH

## Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: Responders *versus* nonresponders

Trygve Ljung, Sofie Lundberg, Mark Varsanyi, Catharina Johansson, Peter T Schmidt, Max Herulf, Jon O Lundberg, Per M Hellström

Trygve Ljung, Sofie Lundberg, Mark Varsanyi, Catharina Johansson, Peter Thelin Schmidt, Per M Hellström, Department of Gastroenterology and Hepatology, Karolinska University Hospital, Site Solna, Stockholm, Sweden  
Max Herulf, Jon O Lundberg, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden  
Supported by the Swedish Research Council, Novo Nordisk and the Bengt Ihre fund

Correspondence to: Trygve Ljung, MD, PhD, Department of Gastroenterology and Hepatology, Karolinska University Hospital, Site Solna, SE-171 76 Stockholm, Sweden. trygve.ljung@hotmail.com  
Telephone: +46-8-51773877 Fax: +46-8-51772058  
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### Abstract

**AIM:** To explore rectal nitric oxide (NO) as biomarker of treatment response in ulcerative colitis (UC) and Crohn's disease (CD), and examine relationships between rectal NO, mucosal expression of NO synthases (NOS), and pro-inflammatory cytokines.

**METHODS:** Twenty-two patients with UC and 24 with CD were monitored during steroid treatment. Rectal NO levels were measured and clinical activities were assessed on days 1, 3, 7 and 28. Mucosal presence of NOS and pro-inflammatory cytokines were analyzed by immunohistochemistry and RT-PCR.

**RESULTS:** Active UC and CD displayed markedly increased rectal NO levels ( $10950 \pm 7610$  and  $5040 \pm 1280$  parts per billion (ppb), respectively) as compared with the controls ( $154 \pm 71$  ppb,  $P < 0.001$ ). Rectal NO correlated weakly with disease activity in both UC and CD ( $r = 0.34$  for UC and  $r = 0.48$  for CD,  $P < 0.01$ ). In 12 patients, a steroid-refractory course led to colectomy. These patients had only slightly increased NO levels (UC:  $620 \pm 270$  ppb; CD:  $1260 \pm 550$  ppb) compared to those with a therapeutic response (UC:  $18860 \pm 530$  ppb,  $P < 0.001$ ; CD:  $10060 \pm 3200$  ppb,  $P < 0.05$ ).

**CONCLUSION:** Rectal NO level is a useful biomarker of treatment response in IBD as low NO levels predicts a poor clinical response to steroid treatment.

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**Key words:** Crohn's disease; Cytokines; Nitric oxide;

### INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory diseases sharing a clinical course with flares of disease, characterized by an increase of symptoms due to increased inflammatory activity of the intestinal mucosa. Symptom-based clinical activity indices are today's standard methods applied to monitor disease activity in clinical trials, but rarely used in clinical practice. Available indices have been criticized for depending almost exclusively on clinical features that are often subjective. The use of systemic markers of inflammation, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count and white blood cell (WBC) count are commonly used in clinical practice, but correlation to ongoing intestinal inflammation is poor<sup>[1,2]</sup>. An approach of direct assessment of mucosal inflammation has been promising, such as measurements of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ <sup>[3]</sup> and intestinal permeability tests<sup>[4]</sup>. The implementation of these tests is, however, restricted by a complicated analysis. The development of a more feasible objective marker of mucosal inflammation is, therefore, warranted. Data on local measurements of inflammatory products such as calprotectin, lactoferrin and nitric oxide (NO) in UC and CD have been promising<sup>[5]</sup>.

We use a minimally invasive method employing chemiluminescence to measure rectal NO levels in conditions with inflamed intestinal mucosa<sup>[6,7]</sup>. Increased NO generation has been demonstrated in both UC and CD<sup>[6,8,9]</sup>. The excessive formation of NO is elaborated by inducible nitric oxide synthase (iNOS)<sup>[10,11]</sup>. Some groups have also reported increased endothelial NOS (eNOS) activity in inflamed mucosa in patients with UC<sup>[12]</sup> and iNOS expression restricted to epithelial cells<sup>[13,14]</sup>. Other groups have demonstrated iNOS expression also in

macrophages and granulocytes of the lamina propria<sup>[10,11]</sup>.

The activation of iNOS is dependent on the transcription factor nuclear factor (NF)- $\kappa$ B, which is activated by bacterial products (e.g., endotoxins) and pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and interferon (INF)- $\gamma$ . These pro-inflammatory cytokines act in synergy to stimulate NO production<sup>[15]</sup>. The aim of the present study was to investigate rectal NO levels as a biomarker of response in UC and CD during glucocorticosteroid (GCS) treatment, using established clinical indices as gold standard for clinical activity. We also assessed mucosal immunoreactivity to the cytokines TNF- $\alpha$ , IL-1 $\beta$  and INF- $\gamma$  as well as activation of eNOS, iNOS and neuronal NOS (nNOS).

## MATERIALS AND METHODS

### Patients

Patients treated with prednisolone (0.5-1 mg/kg orally) at the Karolinska University Hospital for active UC or CD were eligible to enter this study. Forty-six consecutive patients, 22 with UC and 24 with CD, diagnosed by conventional endoscopic, radiological and histological criteria of IBD<sup>[6]</sup> were recruited to this study. Diagnosis of UC or CD was confirmed for all included patients at follow-up one-year after completion of the study. The majority of patients had concomitant medication with aminosalicylates, whereas no patient was on immunomodulators at baseline (Table 1).

The patients were studied at four different occasions during the first month of treatment, before onset of prednisolone treatment (d 1), and at follow-up during ongoing treatment on days 3, 7 and 28. A flexible sigmoidoscopy was performed at baseline, and at all follow-up visits. Disease activity was assessed using the Disease Activity Index (DAI)<sup>[17]</sup> for the patients with UC. The Harvey-Bradshaw Index (HBI)<sup>[18]</sup> was used for the patients with CD. The endoscopic classification was done according to the DAI score also for the CD patients.

At the last visit (d 28), the patients were divided into responders (remission) and non-responders (no remission) for further subgroup analysis. Remission was defined as DAI  $\leq$  2 in UC, and HBI  $\leq$  4 in CD, whereas non-responders were defined as DAI  $\geq$  3 in UC and HBI  $\geq$  5 in CD, respectively.

Two different control groups were used: the control for immunohistochemistry analyses consisted of six individuals undergone control colonoscopy after prior polypectomy, and the control for rectal NO consisted of 25 healthy volunteers with no history of gastrointestinal symptoms or abdominal surgery.

The study was approved by the Karolinska Institutet Ethics Committee. Written informed consent was taken from all patients.

### Determination of rectal NO levels

NO was measured with a chemiluminescence analyzer (CLD 700, Eco Physics, Dürnten, Switzerland). The detection limit for NO was 1 part per billion (ppb). The analyzer was calibrated at known concentrations (100-10000 ppb) of NO in nitrogen gas (AGA, Lidingö,

**Table 1 Patient characteristics**

	Ulcerative colitis (n = 22)	Crohn's disease (n = 24)
Sex (M/F)	13/9	14/10
Age (yr) (mean and range)	41 (18 - 78)	42 (20 - 69)
Duration (yr) (mean and range)	7.4 (0 - 30)	9 (0 - 28)
Smoking habits (n)		
Active	2	3
Former	6	8
Non-smoker	7	9
Unknown	7	4
Concomitant medication (n)		
Aminosalicylates	18	12
Local steroids	6	2
Site of disease		
Ileocolonic		24
Rectum	3	
Proctosigmoiditis	6	
Extensive/total	13	
Disease activity index (mean and range)	9.4 (5 - 12)	
Harvey-Bradshaw index (mean and range)		14.9 (4 - 29)
Endoscopic score (mean and range)	2.2 (1-3)	2.1 (0-3)

Sweden), administered through an electromagnetic flow controller (EnviroNics, Middletown, CT, USA). The chemiluminescence assay is highly specific for NO without interference from other nitrogen oxides<sup>[19]</sup>. For sampling of rectal gas, we applied an all-silicon catheter (Argyle<sup>®</sup>, Sherwood Medical, Tullamore, Ireland) inserted into the rectum, using lubrication gel, free of local anesthetics, to a level 10 cm above the anal sphincter<sup>[7]</sup>. The balloon of the catheter was then inflated with 10 mL of ambient air containing less than 5 ppb of NO, and left for 10 min to equilibrate with gases in the rectum. Thereafter, the gas was withdrawn from the catheter balloon and diluted to a final volume of 50 mL before chemiluminescence analysis with correction for dilution. Analyses were performed within 15 min of sampling<sup>[7]</sup>. In cases where measurements exceeded the upper detection limit, further dilution steps were made in order to measure NO within the calibrated range.

### Immunohistochemistry

Mucosal biopsies were sampled at sigmoidoscopy. Biopsy specimens were always taken in the vicinity of lesions or ulcerations. In case of colectomy (n = 12), the surgical specimens were additionally used for analyses. Biopsies were kept in Histocon (HistoLab, Gothenburg, Sweden) on ice and snap-frozen in liquid nitrogen within 30 min. Approximately 6  $\mu$ m thick cryostat sections were mounted on gelatin-coated glass slides, and stored at -80 °C. Before staining, the slides were thawed at room temperature and subsequently fixed in cold 20 g/L formaldehyde in phosphate-buffered saline (PBS). All following incubation and washing steps were performed in PBS supplemented with 1 g/L saponin (Sigma Chemicals, St Louis, MO, USA) to permeabilize cellular membranes<sup>[20]</sup>. Peroxidase activity was blocked with 3 g/L hydrogen peroxidase and 1 g/L sodium azide in PBS-saponin<sup>[21]</sup>. Human and goat serum

(Vector Laboratories Inc, Burlingame, CA, USA) and avidin/biotin blocking kit (Vector Laboratories) were used to block unspecific bindings. The sections were incubated overnight at 4°C with mouse monoclonal antibodies to iNOS (NOS-IN 20 mg/L, Sigma), nNOS (NOS-BI 73 mg/L, Sigma), eNOS (NOS-EI 17 mg/L, Sigma), IL-1 $\beta$  (2D8 1.4 mg/L, ImmunoKontakt, Abingdon, Oxon, UK), TNF- $\alpha$  (Mab I 10 mg/L + Mab II 14 mg/L, Pharmigen, San Diego, CA, USA), and to INF- $\gamma$  (7-B6- 10 mg/L + 1-DIK 10 mg/L, Mabtech AB, Nacka, Sweden). Mouse IgG<sub>1</sub> (28 mg/L, Dako, Glostrup, Denmark) served as an isotype-matched negative control. Biotinylated goat anti-mouse IgG<sub>1</sub> (4 mg/L, Caltag laboratories, Burlingame, CA, USA) was used as secondary antibody, except for the eNOS staining (an IgA antibody) where it was substituted with biotinylated goat anti-mouse immunoglobulin (11 mg/L, Dako). The biotinylated secondary antibodies were followed by horse-radish peroxidase-conjugated avidin/biotin-complex (Vectastain, ABC-elite, Vector Laboratories). Positive peroxidase staining was developed with 3, 3'-diaminobenzidine (DAB peroxidase substrate kit, Vector Laboratories) and counterstained with haematoxylin (Histolab).

#### Quantification of immunohistochemical staining

All microscopic evaluations were performed by one investigator (T.L.) who was blinded to the clinical data as well as analyzed parameters (eNOS, nNOS, iNOS, TNF- $\alpha$ , IL-1 $\beta$ , INF- $\gamma$  and IgG<sub>1</sub>). To validate the observer's quantification, eight randomly chosen sections were additionally analyzed by a second observer (C. J). Absolute correlation was seen between the two observers. Sections were analyzed using light microscope (Nikon Ltd, Tokyo, Japan). For each section, three different grid areas rich in positive cells of satisfactory technical quality were chosen for quantitative analysis of NOS and cytokine immunohistochemistry. Due to some background staining of the epithelial cells, we restricted our quantitative analysis to the lamina propria. For each area, the number of positive cells was counted at high-power magnification ( $\times 400$ ), thereafter the total number of positive cells was divided with the total grid area. For each section, the result was expressed as the mean number of positive cells per one grid area. For comparison, the percentage of positive cells in the lamina propria was also calculated. A high correlation between immunohistochemistry quantification expressed as positive cells per grid area and percentage positive cells of total cell number in lamina propria was observed ( $r = 0.98$ ,  $P < 0.001$ ).

#### Measurement of iNOS gene expression

Colonic biopsies were taken from four patients with CD and two with UC. The biopsies were collected from both normal and inflamed parts of the colon in each patient. Tissue samples were immediately placed in RNAlater (Qiagen, Hilden, Germany), stored for 24 h at 2-8°C and then at -80°C.

After thawing, total RNA was isolated from the biopsies using the RNeasy mini kit (Qiagen). cDNA was synthesized using oligo (dT)<sub>20</sub> primers and SuperScript III

Table 2A Disease activity index, UC patients

t/d	All (n = 22)	Responder (n = 10)	Non-responder (n = 12)	Operated (n = 5)
1	9.4 (5-12)	9.5 (5-12)	9.4 (5-12)	11.2 (10-12)
3	7.4 (2-12)	7.1 (2-10)	7.6 (5-12)	12
7	5.7 (2-9)	4.8 (2-7)	7.2 (5-9)	NA
28	2.5 (0-8)	0.8 (0-2)	5 (3-8)	NA

Responder (DAI  $\leq 2$ ); non-responder (DAI  $\geq 3$ ).

Table 2B Harvey-Bradshaw index, CD patients

t/d	All (n = 24)	Responder (n = 8)	Non responder (n = 16)	Operated (n = 7)
1	14.9 (4 - 29)	12.8 (4 - 26)	15.9 (9 - 29)	16.9 (11 - 29)
3	11.2 (2 - 22)	7.9 (2 - 13)	12.9 (2 - 22)	16.2 (11 - 22)
7	7.7 (1 - 19)	5.8 (1 - 19)	9.6 (2 - 13)	11.5 (10 - 13)
28	4.2 (1 - 9)	1.9 (1 - 3)	6.9 (4 - 9)	NA

Responder (HBI  $\leq 4$ ); non-responder (HBI  $\geq 5$ ).

enzyme (Invitrogen, Carlsbad, CA, USA). Semi-quantitative RT-PCR was performed using cDNA normalized against the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products obtained with primers specific for the human iNOS gene (sense 5'-CCAAAAGGGTCATCATCTCT-3'; antisense 5'-CCTGCTTACCACCTTCTTG-3') (GenBank accession number AF049656) in the linear amplification range (39 to 45 cycles) were electrophoresed on a 15 g/L agarose gel, photographed and bands quantified (Kodak Gel Logic 100 Imaging System, Rochester, NY, USA).

#### Statistical analysis

For statistical analysis and graph plotting, GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used. Data were expressed as mean  $\pm$  SE, and range where appropriate. Groups of independent data were compared using the Mann-Whitney  $U$  test. Intra-group variation was analyzed with the paired Wilcoxon test. Correlation coefficients between different analyses were calculated using the Spearman rank-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Clinical activity

Baseline DAI or HBI scores did not differ significantly between steroid responding *versus* non-responding patients and did not predict clinical outcome. On d 28, 45% (10/22) patients with UC and 33% (8/24) patients with CD were in remission (DAI  $\leq 2$  and HBI  $\leq 4$ , respectively) (Table 2). In 26.08% (12/46) patients (5 UC, 7 CD), colectomy was carried out on d 2-26 due to steroid-refractory severe disease. The DAI and HBI scores in this subgroup did not differ significantly on d 1 between subsequently operated patients and those responding to treatment (DAI:  $9.5 \pm 0.7$  *vs*  $11.2 \pm 0.5$  for responding and operated UC patients; and HBI:  $12.8 \pm 2.3$  *vs*  $16.9 \pm 2.3$  for responding and operated

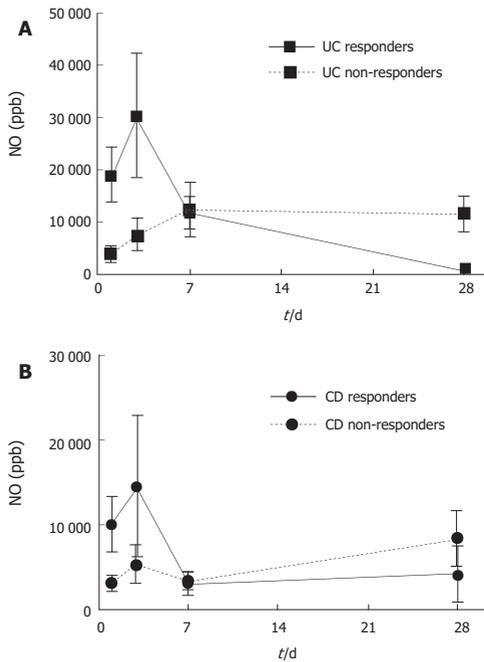


Figure 1 A: Rectal NO levels in UC; B: Rectal NO levels in CD.

CD patients) (Table 2).

#### Rectal NO levels

On d 1, patients with active UC and CD had greatly increased rectal NO levels ( $10950 \pm 7610$  and  $5040 \pm 1280$  ppb, respectively) as compared with the controls ( $154 \pm 71$  ppb, all  $P < 0.001$ ). Repeat measurements of rectal NO showed a numerical increase on d 3 compared to d 1, after which a decrease was seen (Figure 1). This pattern of NO release was mainly attributed to the patients responding to steroid treatment, whereas non-responders displayed a less prominent increase on day 3, and showed no subsequent decrease (Figure 1). For steroid-responding patients, rectal NO levels decreased significantly between d 1 and 28 (from  $18860 \pm 5390$  to  $850 \pm 450$  ppb in UC, and  $10060 \pm 3200$  to  $4130 \pm 3380$  ppb in CD,  $P < 0.001$  and  $P < 0.05$ , respectively).

Rectal NO levels were correlated weakly with clinical activity scores of the whole study population, DAI for UC ( $r = 0.34$ ,  $P < 0.01$ ), and HBI for CD ( $r = 0.48$ ,  $P < 0.01$ ). However, the association was clear-cut in the group of responders to treatment ( $r = 0.72$  for UC and  $r = 0.64$  for CD, all  $P < 0.001$ ).

A different rectal NO pattern was seen in the subgroup of patients in whom colectomy was carried out. The rectal NO levels of these patients were significantly lower at baseline ( $620 \pm 270$  ppb in UC, and  $1260 \pm 550$  ppb in CD) than corresponding values in the patients with a treatment response ( $P < 0.001$  for UC and  $P < 0.05$  for

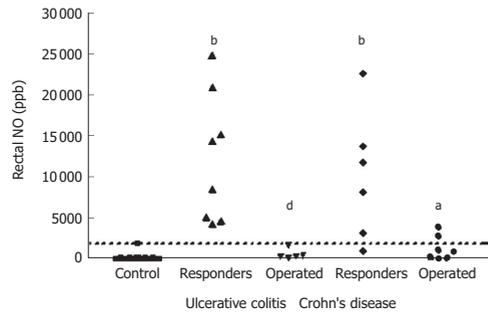


Figure 2 Rectal NO levels in the treatment of UC and CD patients on d 1. <sup>b</sup> $P < 0.001$  vs Control; <sup>a</sup> $P < 0.01$ , <sup>d</sup> $P < 0.05$  vs patients responding to treatment.

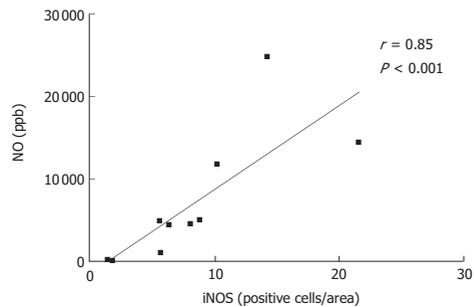


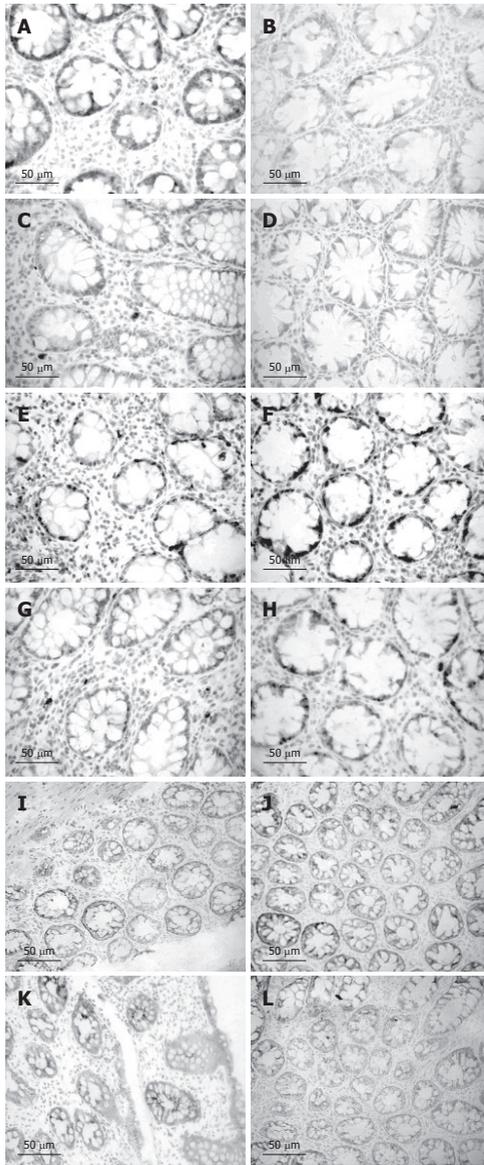
Figure 3 Correlation between mucosal iNOS-expressing cells and rectal NO levels in patients with UC.

CD) (Figure 2). Applying a cut-off level of rectal NO at 2000 ppb in combination with  $\text{DAI} \geq 10$  for patients with UC identified all five patients with a steroid-refractory disease, leading to colectomy within 7 d. Whereas none of the UC patients responding to treatment combined rectal NO  $\leq 2000$  ppb and  $\text{DAI} \geq 10$ . For CD patients, the same cut-off of NO  $\leq 2000$  ppb in combination with  $\text{HBI} \geq 10$  detected 5 of 7 patients subsequently operated, and was seen in 3 CD patients not leading to surgery.

#### Expression of NO synthases and pro-inflammatory cytokines

The number of iNOS-expressing cells, as judged by immunohistochemistry, was significantly higher in patients with UC and CD on d 1 as compared with the healthy controls (all  $P < 0.001$ ). Furthermore, the number of iNOS-positive cells decreased between d 1 and 28 (Figure 4), reaching a borderline significance by pooling UC and CD patients ( $P = 0.064$ ). The number of iNOS-positive cells was obviously correlated with rectal NO levels in patients with UC ( $r = 0.53$ ,  $P < 0.01$ ). This association was strengthened by analyzing the patients responding to treatment ( $r = 0.85$ ,  $P < 0.01$ ) (Figure 3).

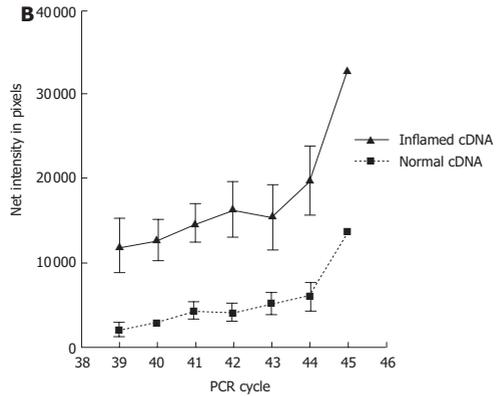
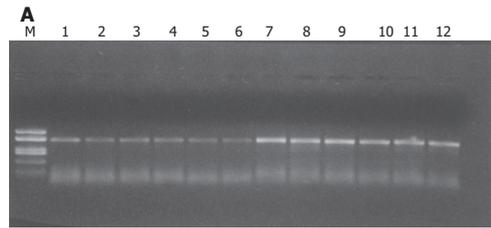
However, no correlation was found between number of iNOS-positive cells and rectal NO levels in patients with CD. The majority of iNOS-positive cells in lamina



**Figure 4** A Colonic histology of CD patients (A, C, E, G) before and (B, D, F, H) after treatment on d 28. Antibodies against iNOS (A, B), TNF- $\alpha$  (C, D), IL-1 $\beta$  (E, F) and INF- $\gamma$  (G, H); Colonic histology of healthy control. Antibodies against iNOS (I), TNF- $\alpha$  (J), IL-1 $\beta$  (K) and INF- $\gamma$  (L).

propria had the morphology of polymorphonuclear leukocytes (Figure 4).

Semi-quantitative RT-PCR showed over-expression of iNOS mRNA in the inflamed compared to normal parts of the mucosa ( $P < 0.05$ ) (Figure 5). Presence of eNOS-positive cells was abundant in all sections and localized



**Figure 5** A: Semi-quantitative PCR iNOS gene of cDNA from non-inflamed (lanes 1-6), and inflamed (lanes 7-12) colon biopsies from a patient with ulcerative colitis; B: Difference in semi-quantitative PCR iNOS gene expression.

to the epithelium, but showed no temporal relationship with rectal NO levels. Recovery of nNOS staining was slight and, when occurring, mostly seen in the submucosa in nerve cells and randomly in mononuclear cells of the lamina propria.

TNF- $\alpha$ , IL-1 $\beta$  and INF- $\gamma$  expressions, as judged by immunohistochemistry, were restricted to mononuclear cells in the lamina propria. IL-1 $\beta$  expression was significantly increased on d 1 compared to healthy controls ( $P < 0.05$ ), whereas TNF- $\alpha$  and INF- $\gamma$  were not. However, both TNF- $\alpha$  and IL-1 $\beta$  decreased numerically between d 1 and 28 for UC and CD (Figure 4). By pooling data from UC and CD patients, a significant reduction was seen ( $P < 0.05$  for both TNF- $\alpha$  and IL-1 $\beta$  immunoreactivity). INF- $\gamma$  did not change between d 1 and 28. A marked increase of iNOS-expressing cells was correlated with TNF- $\alpha$  expression in both UC ( $r = 0.46$ ,  $P < 0.05$ ) and CD ( $r = 0.44$ ,  $P < 0.05$ ). By pooling data for UC and CD, a weak correlation was detected for IL-1 $\beta$  ( $r = 0.33$ ,  $P < 0.05$ ). No correlation was seen between the numbers of iNOS and INF- $\gamma$ -expressing cells.

## DISCUSSION

This study provide evidence suggesting that the greatly increased rectal NO levels seen in active UC and CD are elaborated by high iNOS activity. In our study, a strong correlation was seen between rectal NO levels and clinical

activity indices in patients responding to steroid treatment. A relationship between ongoing inflammatory activity and increased NO production in the gut is well established<sup>[6,8]</sup>. In agreement with this, we found a correlation with clinical disease activity indices in both UC and CD.

Taking into account the relatively low number of patients recruited, the subgroup analysis have to be interpreted with caution. In the non-responding group, no correlation was seen between rectal NO and clinical activity indices. This is in part explained by the aberrant rectal NO pattern in the 12 patients who subsequently underwent colectomy due to severe disease. The finding that baseline rectal NO  $\leq$  2000 ppb in severe UC detected all patients in need of surgery within 7 d due to steroid-refractory disease makes it tempting to suggest rectal NO measurement as a predictive marker of the need of colectomy in active UC. In CD patients, rectal NO seems to be a less strong marker for steroid-response, which is in agreement with previous studies demonstrating a 70% endoscopic remission post-treatment in UC as compared with only 13% endoscopic remission in CD<sup>[22,23]</sup>, as well as a recent study by Costa *et al.*<sup>[24]</sup> showing that fecal calprotectin is a stronger marker for relapse in UC than in CD<sup>[24]</sup>.

Due to the high affinity of NO to hemoglobin<sup>[25]</sup>, one may argue that luminal NO is scavenged by blood in the colon, which should cause falsely low rectal NO levels in the most severe UC and CD cases. This is however unlikely since other severe cases in our group displayed high rectal NO levels in the presence of overt rectal bleeding.

The role of NO in intestinal inflammation is unclear, as both pro-inflammatory and tissue-protective properties have been demonstrated<sup>[26]</sup>. Our observation that high rectal NO levels at the first visit was associated with a favorable clinical outcome is consistent with the idea that NO may act as an endogenous inhibitor of an aggregated immune response. Tissue-protective properties of NO have been shown in animal models of colitis<sup>[27]</sup>. The finding that NO production in collagenous colitis, a chronic inflammatory bowel disease without mucosal cell damage<sup>[28]</sup>, might be even greater than in active IBD supports the concept of NO acting as a modulator of inflammatory activity in the gut. An uncontrolled chronic inflammation could, however, lead to an intracellular depletion of L-arginine, during which NOS will produce O<sub>2</sub> instead of NO, O<sub>2</sub> will then immediately react with NO to form ONOO<sup>-</sup>, considered to be highly cytotoxic<sup>[29]</sup>.

Genetic polymorphisms of iNOS might be a plausible explanation for the different rectal NO patterns seen in the group of responding *versus* non-responding patients. iNOS polymorphism has previously been associated with outcome variables in different diagnoses<sup>[30-32]</sup>.

There is substantial evidence pointing towards iNOS-expressing epithelial cells lining the mucosa as a major site of NO production in intestinal inflammation<sup>[13-15]</sup>. We could not confirm this finding in our present study as we encountered unspecific staining of the epithelial cells, but we consider the correlation between the number of iNOS-positive cells in lamina propria and the production of NO, measured as rectal NO levels, as circumstantial evidence, suggesting the importance of these cells in

the production of the increased NO levels seen in active UC and CD. This is supported not only by earlier studies showing iNOS-positive macrophages and granulocytes in the mucosa in IBD<sup>[11]</sup>, but also by our present finding of a molecular activation of iNOS within the tissue. Our morphological examination of iNOS-positive cells ascribes polymorphonuclear leukocytes as a plausible cellular source of NO production.

Whether the constitutive forms of NOS contribute to the increased NO production in IBD is unclear. For eNOS or nNOS, we could not detect any differences in staining between healthy controls, active IBD or IBD in remission. Little information is available on possible changes in eNOS and nNOS in IBD in literature. One previous study reports findings in line with our results<sup>[14]</sup>, i.e. activation of mainly iNOS, whereas another group claims specific changes in eNOS and nNOS expression in UC<sup>[12]</sup>.

Determination of tissue cytokine levels in IBD has resulted in disparate results. In general, pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and INF- $\gamma$ ) are believed to have a regulatory function in the activated immune response in IBD. Clearly the last years' development has pin-pointed the key role of TNF- $\alpha$  in CD<sup>[3,21,33]</sup>. In line with some previous studies, we failed to show increased TNF- $\alpha$  expression in IBD compared to controls<sup>[34,35]</sup>. However, we found a decrease in TNF- $\alpha$  expression as a response to treatment, supporting data demonstrating an association between TNF- $\alpha$  expression and active IBD<sup>[3,36,37]</sup>. Our data showing increased IL-1 $\beta$  expression in active IBD compared to healthy controls are consistent with earlier studies providing evidence that IL-1 $\beta$  has a central role in the mucosal inflammation as seen in IBD<sup>[34,36,37]</sup>. In our study, we found no support for INF- $\gamma$  as a marker of disease activity in IBD.

We found a correlation between TNF- $\alpha$  and IL-1 $\beta$  expression and iNOS expression, supporting the concept that these cytokines may have a role in inducing NO production<sup>[15]</sup>.

In summary, our study shows that active UC and CD are associated with highly increased rectal NO levels. Rectal NO levels decreases in response to steroid treatment, hence offering a plausible and feasible objective method to monitor disease activity in IBD. Low rectal NO levels  $\leq$  2000 ppb might be a predictive marker for steroid-refractory IBD requiring acute colectomy. Furthermore, our data suggest that the major part of the increased NO production seen in active IBD is elaborated by the inducible form of NOS.

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III



## Integrin $\alpha 2\beta 1$ Regulates Neutrophil Recruitment and Inflammatory Activity in Experimental Colitis in Mice

Sofie Lundberg,\* Johan Lindholm,† Lennart Lindbom,‡ Per M. Hellström,\* and Joachim Werr†

**Background:** Human inflammatory bowel disease (e.g., Crohn's disease and ulcerative colitis), is associated with leukocyte accumulation in the inflamed intestinal tissue. Recent studies strongly suggest a role of  $\beta 1$  integrin receptors in regulating tissue damage and disease symptoms related to inflammatory bowel disease. The aim of this study was to investigate the role of the collagen-binding  $\alpha 2\beta 1$  integrin (CD49b/CD29) in dextran sodium sulfate-induced colitis in mice.

**Methods:** Colitis was induced in mice through oral administration of 2% dextran sodium sulfate in drinking water. Rectal administration of anti- $\alpha 2$ -monoclonal antibody (mAb) in 1 group was compared with oral treatment with betamethasone in another group and rectal administration of a control antibody in a third group. Clinical and histological signs of colitis, neutrophil infiltration into the colon mucosa, and gene expression of metalloproteinases were assessed.

**Results:** Rectal administration of anti- $\alpha 2$ -mAb was found to significantly reduce weight loss from  $13.5\% \pm 6.5\%$  to  $2.2\% \pm 0.2\%$  ( $P = 0.013$  versus control mAb) and mucosal neutrophil infiltration from  $47.2 \pm 10.0$  to  $6.6 \pm 8.0$  neutrophils per counted area ( $P < 0.05$  versus control mAb). Metalloproteinase gene expression was suppressed through anti- $\alpha 2$ -mAb treatment. The protective effect against colitis seen after anti- $\alpha 2\beta 1$  integrin treatment was found to be favorable to the effect seen after high-dose oral betamethasone.

**Conclusions:** We demonstrate an alleviating action of the collagen-binding  $\alpha 2\beta 1$  integrin in experimental colitis in mice and suggest that this effect is mediated by inhibition of neutrophil migration and activation. Local administration of function-blocking antibodies against integrin  $\alpha 2\beta 1$  may provide novel avenues to treat inflammatory bowel disease.

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From the \*Department of Medicine, Unit of Gastroenterology and Hepatology, Karolinska Institutet, Karolinska University Hospital, Stockholm;

†Department of Pathology and Cytology, Unit of General Pathology, Karolinska University Hospital, Stockholm; and ‡Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden.

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Reprints: Sofie Lundberg, MSc, Department of Medicine, Unit of Gastroenterology and Hepatology, Karolinska Institutet Karolinska University Hospital, Solna SE-171 76 Stockholm, Sweden (e-mail: Sofie.Lundberg@ki.se)

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Leukocyte recruitment and leukocyte-mediated tissue injury are important parts of the pathophysiology of human inflammatory bowel disease (IBD).<sup>1–3</sup> Guided by chemotactic signals, leukocytes escape from the vasculature and migrate through the extravascular space to reach sites of infection or tissue injury.<sup>4,5</sup> For this purpose, the cells need to establish transient contacts with endothelial ligands and extracellular matrix (ECM) proteins, a coordinated sequence of events that is precisely regulated by the activation and expression of endothelial and leukocytic cell surface adhesion molecules. The  $\beta 1$  integrin family comprises at least 10 different receptors that specifically bind to ECM proteins such as collagen, fibronectin, and laminin.<sup>6–8</sup> Members of the  $\beta 1$  integrin family have been shown to be upregulated in leukocytes during emigration and extravascular migration and appear to be critically involved in regulating the immune cell trafficking from blood to tissue.<sup>9–11</sup>

Recent reports have documented a role for  $\alpha 1\beta 1$  and  $\alpha 4\beta 1$  integrin receptors in IBD and concluded that these receptors have an important regulatory function in the initiation and perpetuation of the disease.<sup>12–14</sup> In addition, antagonistic therapy directed against  $\alpha 4\beta 1$  integrin has been shown to be effective in combating Crohn's disease in human clinical trials.<sup>15,16</sup> Thus, specific subsets of the  $\beta 1$  integrins are emerging as targets for anti-inflammatory interventions in IBD.

The  $\alpha 2\beta 1$  integrin is a collagen receptor widely expressed on tissue cells, whereas expression on leukocytes is seen only on specific activation. Neutrophils were originally considered to lack  $\alpha 2\beta 1$ , but later findings show that surface expression is induced in these cells in conjunction with their emigration from the vasculature and suggest an important role for the receptor in regulating neutrophil extravascular motility.<sup>11,17</sup> From these findings, we hypothesized that the  $\alpha 2\beta 1$  integrin also may play a regulatory function in IBD. We treated mice with dextran sodium sulfate (DSS)-induced colitis with function-blocking antibodies directed against the  $\alpha 2$  integrin subunit. Antibodies were administered via rectal injections according to a daily schedule. The purpose of our study was to find out whether administration of anti- $\alpha 2$  antibody could mitigate development of colitis, as demonstrated by prevention of weight loss, and histologically detected reduction of mucosal lesions and neutrophil accumulation in

the colonic mucosa. The gene expression of several matrix metalloproteinases (MMPs) relevant for colitis was significantly downregulated by the antibody against the  $\alpha 2$  integrin subunit, indicating that the integrin receptor may play a regulatory function in the expression of MMPs through adhesion-dependent mechanisms.

## MATERIALS AND METHODS

### Mice

The study was approved by the regional ethics committee for the humane use of research animals in northern Stockholm (diary number 432-04). Female BALB/c mice, 6 weeks old, weighing 15 to 20 g were purchased from B&K Universal AB (Sollemtuna, Sweden). The mice were housed under standard conditions with free access to commercial food and drinking water. The experiments started after 1 week of acclimatization after arrival at the laboratory.

### Experimental Design

Experiments were conducted in 7-week-old healthy female BALB/c mice. Colitis was induced by 2% DSS (molecular weight, 40 kD; TdB Consultancy, Uppsala, Sweden) dissolved in purified drinking water for 12 days. The mice given DSS were divided into 3 groups consisting of 5 to 8 mice each and were compared with untreated control animals ( $n = 5$ ) that received purified water. One group of mice ( $n = 5$ ) given DSS received betamethasone (Apoteket AB, Stockholm, Sweden), 0.25 mg/kg, in their drinking water.

### Daily Assessment of Colitis

Daily assessment of animals included measurement of drinking water volume and body weight and evaluation of blood in feces and diarrhea.

### Antibodies

Function-blocking monoclonal antibody (mAb) Ha1/29 against the  $\alpha 2$  subunit (CD49b) and the isotype control mAb Ha 4/8 (both hamster anti-rat mAbs from Pharmingen, San Diego, Calif) were used. The antibodies were administered rectally by injection through a catheter. A dose of 20  $\mu$ g antibody in 80  $\mu$ L purified water was administered daily, starting day 0 after DSS and continuing throughout the duration of the experiment. Intraperitoneal injection of antibody was performed in 1 treatment group at days 3, 5, and 8 after DSS treatment started and at a total dose of 80  $\mu$ g in 200  $\mu$ L purified water.

### Histological Assessment of Colitis

All of the animals were killed at day 14, and their colons were removed. Colon length and weight were measured in the fresh specimens. Samples for RNA extraction were obtained from the distal colon. The freshly obtained colon

was then fixated in 4% formaldehyde and embedded in paraffin before staining with hematoxylin and eosin.

Histological quantification of mucosal damage and inflammation was performed along the entire longitudinal sections of the specimens. Specimens and treatment groups were blinded before histological quantification. Histological scoring was performed by the use of 2 independent parameters: percentage of mucosal surface affected by lesions and neutrophil count in mucosal lesions. Lesions were defined as visible damage of the mucosal surface involving more than two thirds of the total mucosal thickness. Cell count of hematoxylin and eosin-stained neutrophils was consistently performed in 3 high-power fields ( $40\times$  lens covering  $0.45\times 0.45$  mm) in the lamina propria in the most distal lesion in each animal. Neutrophils were identified through high magnification of hematoxylin-stained nuclei. Images were obtained with a Nikon Eclipse E800 microscope (Tokyo, Japan).

### Mouse Gene Array

The GEArray Q Series Mouse Nitric Oxide Gene Array was purchased from SuperArray (Bioscience Corp, Frederick, Md). Total RNA was isolated from the 4 groups—healthy control mice, colitis, anti- $\alpha 2$ -mAb-treated, and betamethasone-treated mice—with the use of RNeasy Mini Kit (Qiagen, Hilden, Germany). A total of 4 to 5  $\mu$ g RNA from each group was pooled and used as a template to generate  $^{32}$ P-labeled cDNA probes according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60°C with the SuperArray membrane, which was washed and exposed with the phosphor imager screens for 72 hours. The phosphor imager screen was scanned by a Fujifilm BAS-2500 machine, saved as a TIFF file, and analyzed with SuperArray's GEArray Expression Analysis Suite program. The averages of 2 glycerol aldehyde phosphate dehydrogenase and 2  $\beta$ -actin spots were used as positive controls and set as baseline values with which the signal intensity of other spots was compared. Using these normalized data, we compared the signal intensity from the membranes using the GEArray analyzer program.

### Data Analysis

Values are given as mean  $\pm$  SEM. Statistical analysis was performed by the use of 1-way analysis of variance unpaired  $t$  test and Kruskal-Wallis test when appropriate. Statistical significance was defined as  $P < 0.05$ .

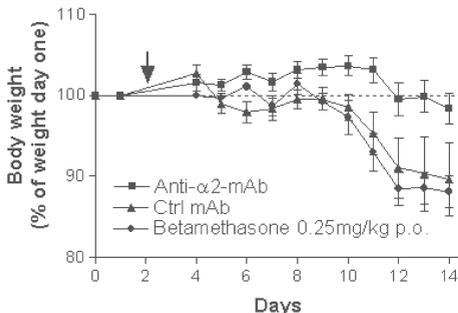
## RESULTS

### Effect of Anti- $\alpha 2$ mAb on Colitis-Dependent Weight Loss

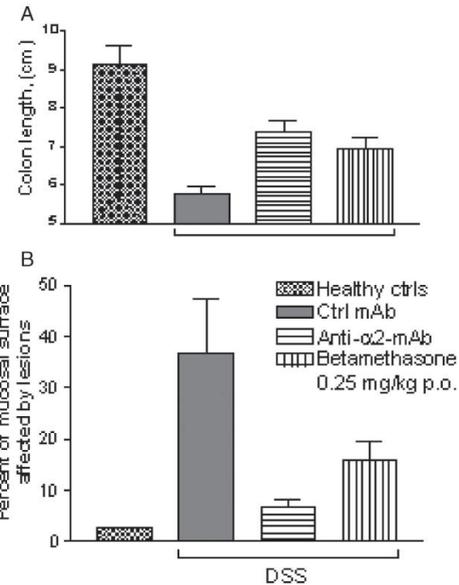
Function-blocking mAb Ha1/29 directed against the  $\alpha 2$  subunit (CD49b) of the  $\alpha 2\beta 1$  integrin (CD49b/CD29) was used for the experiments. Rectal administration of anti- $\alpha 2$ -mAb in 1 group was compared with oral treatment with betamethasone

in another group and rectal administration of a control antibody in a third group. The 3 separate treatment groups of animals had comparable body weights of  $16.7 \pm 1.1$  g ( $n = 8$ ),  $17.3 \pm 0.9$  g ( $n = 5$ ), and  $17.4 \pm 1.0$  g ( $n = 6$ ) when entering the study. The 3 groups were monitored daily for 14 days. Of several clinical parameters monitored (diarrhea, fecal blood, and weight loss), the most prominent clinical sign of disease was weight loss (Fig. 1). As shown in Figure 1, animals that received DSS and a control antibody lost  $13.5\% \pm 6.5\%$  of their body weight during the test period. Rectal administration of anti- $\alpha 2$ -mAb completely prevented weight loss ( $2.2\% \pm 0.2\%$  decrease in weight;  $P = 0.013$  versus control mAb) and thus significantly reduced the severity of disease. Healthy animals showed a stable and constant weight curve during the test period of 12 days ( $3.1\% \pm 0.4\%$  increase in weight). There were also subjective signs of the efficacy of antibody treatment accompanying the effect on clinical parameters. Animals treated with anti- $\alpha 2$ -mAb preserved their activity level and their fur-cleaning ability, whereas animals treated only with control antibody lost their activity level and cleaning behavior during the latter phase of the test period. To investigate a potential additive effect of both rectal and intraperitoneal administration of the antibody, 1 treatment group received rectal anti- $\alpha 2$ -mAb according to the daily schedule together with intraperitoneal injections of the antibody on days 3, 5, and 8. Combined rectal and intraperitoneal administration of antibody prevented weight loss as effectively as the rectal antibody alone.

The effect of anti- $\alpha 2$ -mAb administration on DSS colitis was compared with the effect of oral high-dose betamethasone administered to the animals through their drinking water. Interestingly, as shown in Figure 1, betamethasone



**FIGURE 1.** Anti- $\alpha 2$ -mAb prevents weight loss in DSS-mediated colitis in mice. Before DSS treatment started, body weight was assessed in animals for 2 consecutive days (days 0 and 1). The animals received DSS in their drinking water for 12 days (day 2 to 14). Anti- $\alpha 2$ -mAb was administered rectally once per day simultaneously with DSS treatment. Betamethasone was administered through drinking water at a daily dose of 0.25 mg/kg. The arrow indicates the start of treatment with DSS and antibody or betamethasone ( $n = 5$ –9 mice/group).



**FIGURE 2.** DSS colitis is associated with shortening of the colon (a) and extensive mucosal lesions (b). Anti- $\alpha 2$ -mAb resulted in less shortening of the colon and prevented mucosal lesions. The colon was excised after 12 days of treatment with DSS, and the total length of the fresh specimen was measured. Colons were then fixed and stained with hematoxylin and eosin. Longitudinal sections of the distal colon mucosa were analyzed for mucosal lesions ( $n = 5$ –8 mice/group).

treatment did not significantly prevent weight loss induced by DSS colitis during the test period. The weight loss in the betamethasone-treated group was  $11.9 \pm 4.5\%$  and did not differ significantly from the animals treated with DSS alone.

### Effect of Anti- $\alpha 2$ -mAb on Histological Signs of Colitis

Colon length in healthy animals was found to be  $9.1 \pm 1.0$  cm (Fig. 2a). As described in Figure 2a, animals treated with DSS and control antibody showed significant shortening of the colon ( $P < 0.001$ ). The length of the colons of these animals was  $5.8 \pm 0.8$  cm. Treatment with anti- $\alpha 2$ -mAb could significantly reduce DSS-induced shortening of the colon ( $7.4 \pm 0.8$  cm;  $P < 0.05$  versus control mAb). High-dose betamethasone treatment also was found to prevent shortening of the colon ( $6.9 \pm 0.7$  cm;  $P = \text{NS}$  for trend). The DSS-induced colitis was characterized by mucosal lesions in the distal colon (Fig. 2b). Longitudinal sections of the distal colon were analyzed for mucosal lesions. The lengths of all lesions in 1 section were added and divided by the total length of the specimen to obtain the percentage of mucosa affected by

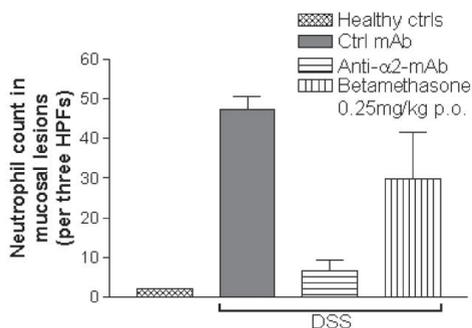
lesions. In DSS-treated animals,  $36.7\% \pm 10.5\%$  of the mucosa analyzed in a longitudinal section of the distal colon was affected by lesions. In animals treated with anti- $\alpha 2$ -mAb,  $6.5\% \pm 1.5\%$  of the mucosa was affected by lesions ( $P = 0.015$  versus control mAb). Betamethasone also protected against mucosal lesions ( $15.8\% \pm 3.7\%$ ), although to a lesser extent than anti- $\alpha 2$ -mAb treatment.

**Effect of Anti- $\alpha 2$ -mAb on Neutrophil Recruitment to Mucosal Lesions**

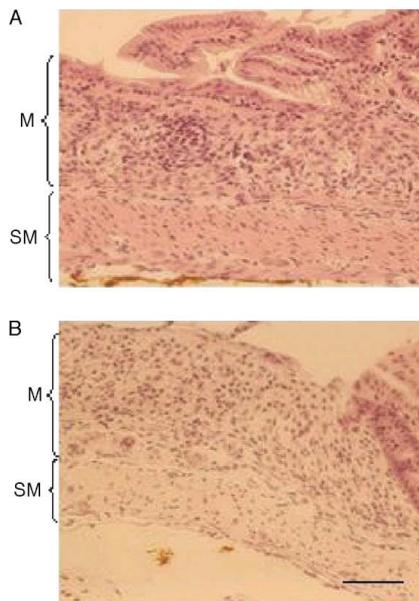
The prevalence of neutrophils in mucosal lesions was assessed by a manual cell count of neutrophils in a defined area within lesions. As shown in Figure 3, anti- $\alpha 2$ -mAb treatment resulted in a dramatic reduction of neutrophil presence in mucosal lesions, from  $47.2 \pm 10.0$  neutrophils (per 3 high-power fields) in the DSS-treated control mAb group to  $6.6 \pm 8.0$  neutrophils in the DSS-treated group receiving anti- $\alpha 2$ -mAb ( $P = 0.007$ ). Betamethasone resulted in some reduction of neutrophil influx ( $29.8 \pm 11.7$  neutrophils;  $P = \text{NS}$  for trend), but not at the same magnitude as seen after anti- $\alpha 2$  treatment. To further clarify the effect of anti- $\alpha 2$ -mAb treatment on neutrophil recruitment, comparative analyses of the distribution of neutrophils within colonic lesions were performed. As shown in the representative images in Figure 4a, transmural infiltration of neutrophils across the entire colonic wall was seen in DSS-treated control mAb animals. In contrast, in anti- $\alpha 2$ -treated animals, neutrophil infiltration was less pronounced (as quantified in the cell count) and located predominantly in the mucosa.

**Effect of Anti- $\alpha 2$ -mAb on Gene Expression of MMPs**

Expressions of MMP genes were analyzed with a GEArray kit. RNA from each treatment group was pooled.



**FIGURE 3.** Neutrophil recruitment to mucosal lesions is impaired by anti- $\alpha 2$ -mAb treatment. The prevalence of neutrophils in mucosal lesions was assessed by manual cell counting of stained cells in 3 high-power fields per lesion ( $40\times$  lens covering  $0.45 \times 0.45$  mm).  $n = 5$  mice per group.

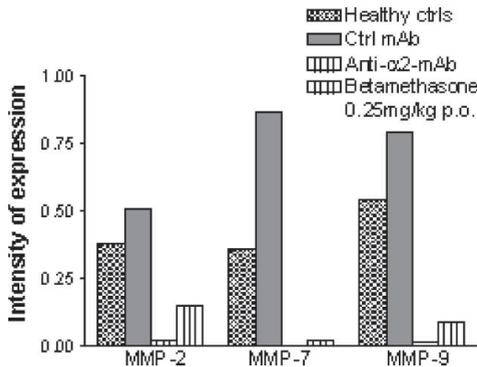


**FIGURE 4.** Images of the distribution of extravasated neutrophils within colonic lesions in DSS-treated animals (a) and anti- $\alpha 2$ -mAb-treated animals (b). Longitudinal sections of the distal colon are shown. Transmural neutrophil infiltration was seen in DSS-treated animals, whereas anti- $\alpha 2$ -treated animals showed less neutrophil infiltration, located predominantly in the mucosa. The images are representative for each treatment group ( $20\times$  lens covering  $0.9 \times 0.9$  mm). M indicates mucosa; SM, submucosa. Bar =  $100 \mu\text{m}$ .

As shown in Figure 5a, DSS-treatment resulted in significantly increased gene expression of MMP-2, -7, and -9 compared with healthy animals (1.3- to 2.4-fold increase versus healthy animals). Administration of anti- $\alpha 2$ -mAb resulted in a complete downregulation of all 3 MMPs to levels below those seen in healthy animals (25.5- to 87.0-fold reduction compared with DSS-treated animals and 19.0- to 54.0-fold reduction compared with healthy controls). Most pronounced was the effect on MMP-7, with a 2.4-fold upregulation in DSS-treated animals and complete suppression below detectable levels after anti- $\alpha 2$ -mAb treatment. A similar pattern of downregulated MMPs was seen after oral treatment with high-dose betamethasone.

**DISCUSSION**

Leukocyte recruitment is a multistep process regulated by adhesion molecule interactions between leukocytes and endothelial cells and ECM proteins.<sup>4,18</sup> Recruitment of



**FIGURE 5.** Anti- $\alpha$ 2 treatment decreases MMP gene expression in mice with DSS colitis. GEArray gene expression analysis was performed for MMP-2, -7, and -9. The averages of 2 glycerol aldehyde phosphate dehydrogenase and 2  $\beta$ -actin spots were used as positive controls and set as baseline values with which the signal intensity of other spots was compared. Material from each treatment group was pooled and then analyzed ( $n = 3$ –5 mice/group).

circulating leukocytes to the intestinal mucosa is a pivotal step in the initiation and perpetuation of IBD.<sup>19</sup> Therapeutic compounds directed against specific cell adhesion molecules have been tested as a treatment for Crohn's disease and ulcerative colitis, and to date, at least 4 different cell adhesion molecules have been demonstrated to regulate disease activity in experimental models of colitis.<sup>12–14,19–22</sup> Thus, there is a growing body of evidence that several different cell adhesion molecules could function as specific targets for therapeutic interventions in IBD.

The DSS colitis model is well characterized and involves the activation of both neutrophils and monocytes with minor contributions of lymphocytes in the early phase. In the present study, we assessed the functional role of the collagen-binding integrin receptor  $\alpha$ 2 $\beta$ 1 in DSS colitis in mice. The expression of  $\alpha$ 2 $\beta$ 1 in leukocytes was previously believed to be restricted to activated mononuclear leukocytes but later shown to be induced also in neutrophils on extravasation of these cells from the vasculature.<sup>11,17</sup> We have previously demonstrated a critical role of  $\alpha$ 2 $\beta$ 1 integrin in neutrophil migration in extravascular tissue, suggesting a function of the receptor also in the pathogenesis of inflammatory disease.<sup>17</sup>

In this study, we demonstrated a critical role of  $\alpha$ 2 $\beta$ 1 integrin in regulating DSS colitis. Daily rectal administration of a function-blocking antibody against  $\alpha$ 2 $\beta$ 1 integrin could prevent weight loss and significantly reduce histopathological signs of disease in the colon of DSS-treated animals. We identified that disruption of  $\alpha$ 2 $\beta$ 1 integrin-dependent adhesion leads to impaired neutrophil accumulation at sites of

mucosal damage in the colon. Similarly, the extent of inflammatory changes in the colonic mucosa was significantly reduced through blockage of  $\alpha$ 2 $\beta$ 1 integrin.

We also investigated whether blockage of  $\alpha$ 2 $\beta$ 1 integrin affected the expression of MMPs, well known to be involved in the pathophysiology of IBD. MMPs are proteinases involved in the breakdown and remodeling of the ECM under a variety of physiological and pathological conditions. MMP-2 and MMP-9, collectively known as the gelatinases, are particularly important in the pathogenesis of IBD.<sup>23–25</sup> Matrilysin, an MMP-7, has previously been associated with many different tumor types, and recent reports have shown important roles in inflammatory disorders.<sup>26</sup>

Our observation of an upregulation of MMP gene expression in DSS colitis is in line with previous reports of elevated MMP expression in Crohn's disease, ulcerative colitis, and several experimental models of colitis, including DSS colitis.<sup>23,27–29</sup> We observed upregulation of MMP-2, -7, and -9 in DSS-treated animals. Anti- $\alpha$ 2 $\beta$ 1 integrin treatment completely suppressed gene expression of all 3 MMPs below levels seen in healthy animals, indicating that the integrin receptor may play a direct or indirect regulatory function for MMP expression in the inflamed colonic tissue. Betamethasone, although less potent than anti- $\alpha$ 2-mAb, also suppressed expression of all 3 MMPs. Indeed, a recent report demonstrates that  $\alpha$ 2 $\beta$ 1 integrin can modulate MMP activity through adhesion-dependent mechanisms and that disruption of  $\alpha$ 2 $\beta$ 1 integrin adhesion to collagen results in downregulation of MMPs.<sup>30</sup> Thus, our data on MMP suppression through anti- $\alpha$ 2 treatment may indicate that MMPs are regulated in colitis by adhesion-dependent mechanisms involving  $\alpha$ 2 $\beta$ 1 integrin interaction with collagen.

The improvement in colitis seen in mice treated with anti- $\alpha$ 2 antibody compared favorably with that seen after treatment with oral high-dose betamethasone. Although betamethasone could partially protect against mucosal damage and neutrophil influx, it was less effective in preventing weight loss and thus clinical signs of disease. This finding underlines the importance of  $\alpha$ 2 $\beta$ 1 integrin-mediated interactions in the initiation and progression of DSS colitis and indicates that  $\alpha$ 2 $\beta$ 1 is a potentially interesting therapeutic target.

Recent studies have demonstrated a similarly important role for  $\alpha$ 1 $\beta$ 1 integrin and monocytes in DSS colitis.<sup>12,13</sup> In these studies, it was shown that disruption of  $\alpha$ 1 $\beta$ 1 affects both motility and cytokine production in monocytes. Both  $\alpha$ 1 $\beta$ 1- and  $\alpha$ 2 $\beta$ 1 integrins bind to collagen with a preference to collagen type IV and collagen type I, respectively, and their matrix-binding capabilities are carefully regulated.<sup>31</sup> Interestingly, our data and the previous study by Krieglstein et al<sup>12</sup> show that blockage of the collagen-binding integrins  $\alpha$ 1 $\beta$ 1 or  $\alpha$ 2 $\beta$ 1 independently of each other results in significantly reduced disease activity in DSS colitis. In addition, data obtained from clinical trials investigating the therapeutic effect of  $\alpha$ 4

integrin blockage in IBD demonstrate the involvement of additional integrin receptors in the pathogenesis of this disease.<sup>15,16</sup> Thus, to date, at least 3 different  $\beta 1$  integrins have been demonstrated to be involved in regulating IBD or experimental forms of IBD such as DSS colitis. Combined, these observations support the concept that integrins, particularly  $\beta 1$  integrins, play a critical role in both intravascular and extravascular events of leukocyte recruitment in IBD and thus may constitute targets for therapeutic interventions in inflammatory disease.

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IV



# **Integrin-blockade compared to conventional treatment in experimental colitis**

Sofie Lundberg (1), Johan Lindholm (2), Lennart Lindbom (3),  
Per M. Hellström (1) and Joachim Werr (3)

Departments of (1) Medicine, Unit of Gastroenterology and Hepatology, (2) Pathology and Cytology, Unit of General Pathology, Karolinska University Hospital, Solna, and (3) Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

## **Correspondence to:**

Sofie Lundberg, MSc

Department of Medicine, Unit of Gastroenterology  
and Hepatology, Karolinska Institutet, Karolinska  
University Hospital, Solna

SE-171 76 Stockholm, Sweden

Telephone: +46 8 517 722 89, Fax: +46 8 517 711 00

E-mail: Sofie.Lundberg@ki.se

## **Short title:**

Integrin blockade in colitis

## **Keywords:**

Inflammation, Antibodies, Integrins, DSS, and Inflammatory Bowel Disease

## Abstract

**Background:** Inflammatory bowel disease (IBD) is associated with leukocyte infiltration of intestinal tissue. Recent reports demonstrate that inhibition of leukocyte recruitment through anti-integrin-treatment is an effective strategy in the treatment of IBD. Several specific integrin receptors have successfully been targeted through both systemic and local/rectal administration of anti-integrin antibodies. The aim of this study was to compare the therapeutic effect of methotrexate, 5-aminosalicylic acid (5-ASA), and azathioprine with anti-integrin treatment directed against  $\alpha 2$  (CD49b) and  $\alpha 4$  (CD49d) integrin on dextran sodium sulfate (DSS)-induced colitis in mice.

**Methods:** Mice received 2.5% DSS in their drinking water. After 13 days, when the animals had established clinical signs of colitis, one group of animals was killed and their colons examined histologically. The other animals received daily rectal treatment with methotrexate, 5-ASA, anti- $\alpha 2$  or anti- $\alpha 4$  integrin antibody. Azathioprine was given orally. Clinical signs of disease such as diarrhea, weight loss and rectal bleeding were monitored daily. After 19 days all animals were killed, their colons removed and analyzed histologically. The different treatment groups were compared with the animals killed after 13 days and an untreated control group to disclose potential signs of disease remission.

**Results:** Histological analysis of the distal colon revealed sign of remission and less mucosal damage in animals treated with anti- $\alpha 2$  integrin antibody, azathioprine, and methotrexate compared to animals treated with anti- $\alpha 4$  integrin antibody and 5-ASA. Treatments against  $\alpha 2$  and  $\alpha 4$  integrin were effective in alleviating diarrhea and rectal bleeding ( $p < 0.05$ ). Only anti- $\alpha 2$  antibody inhibited colonic shortening ( $p < 0.05$ ), known to be associated with severity of colitis. Of the comparative treatments given, methotrexate was most effective in ameliorating weight loss due to colitis, but did not significantly reduce rectal bleeding and diarrhea.

**Conclusions:** Anti-integrin treatment through rectal administration of anti- $\alpha 2$  or - $\alpha 4$  integrin antibodies reduces clinical and histological signs of colitis in mice. The protective effect against colitis seen after anti-integrin treatment is favourable to that seen after 5-ASA and azathioprine and comparable with a high dose of methotrexate. Thus, our data show that anti- $\alpha 2$  and - $\alpha 4$  integrin treatment are highly potent in the treatment of DSS colitis in mice, superior to 5-ASA as well as azathioprine and methotrexate.

## Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of the gastrointestinal (GI) tract with significant morbidity and limited therapeutic interventions. The current medical treatment of IBD relies on the use of anti-inflammatory and immunosuppressive agents with limited specificity, severe side effects and limited long-term benefits (1, 2). Thus, there is a need for new therapies that are well tolerated and effectively induce remission or alter the chronic course of the disease.

A central feature of IBD is dysregulation of leukocyte recruitment towards the affected tissue. T cells, neutrophils and monocytes have been shown to serve an important role in modulating immune responses and subsequent tissue damage (3-8). Several novel treatment strategies in IBD target leukocytes directly and are designed to alter recruitment and function of these cells in the affected intestinal tissue (7). More specifically, therapies disrupting leukocyte-endothelial and leukocyte-matrix interactions via integrin receptors have shown promising results. In line with this, blockade of specific integrins and their endothelial ligands have shown beneficial effects in animal models of colitis and even in human trials (9).

Integrins are heterodimeric proteins that consist of an  $\alpha$  and a  $\beta$  subunit. Integrin receptors belonging to the common  $\beta 1$  and  $\beta 2$  integrin subunit have been successfully targeted in IBD (10-12).

Counter receptors to integrins belong to the immunoglobulin (Ig) superfamily. Over-expression of several endothelial receptors such as E-selectin, ICAM-1, ICAM-2 and VCAM-1 have been observed in the intestinal mucosa of patients affected with IBD (13-17). Anti-ICAM-1 therapy has been evaluated in several human trials with encouraging results (9). Anti-integrin treatment in IBD is emerging as a novel treatment strategy that can be designed to be more effective than conventional treatment. Thus, there is a need for direct comparisons of conventional versus anti-integrin treatment to better understand the potential of these directed therapies. In this report we use the dextran sodium sulfate (DSS) colitis model in mice to compare the therapeutic effect of conventional treatment with methotrexate, 5-aminosalicylic acid (5-ASA), azathioprine, and anti-integrin treatment against  $\alpha 2$  (CD49b) and  $\alpha 4$  (CD49d) integrin. The DSS model, originally reported by Okayasu et al. (18), is commonly used to investigate

the role of leukocytes in intestinal inflammation. Clinical as well as histological features of the DSS model resemble IBD in humans. The immunopathogenesis of the model involves infiltration of neutrophils, monocytes and lymphocytes (10-12, 19-21). Therefore, we used the DSS model to investigate conventional and anti-integrin treatment in experimental colitis. We found the ameliorating effect against colitis seen after anti-integrin treatment to be favourable to the effect seen after 5-ASA and azathioprine and comparable with methotrexate. Interestingly, anti- $\alpha 2$  integrin treatment results in most histological remission of animals that had already established colitis when treatment started.

Thus, our data show that anti- $\alpha 2$  and - $\alpha 4$  integrin treatment are highly potent in the treatment of DSS colitis in mice with an outcome favourable to 5-ASA, azathioprine, and methotrexate.

## Material and Methods

*Mice.* The study was approved by the Regional Ethics Committee for the Humane use of Research Animals in Northern Stockholm. Thirtyfour female BALB/c mice, weighing 19-21 g were purchased from B&K Universal (Sollentuna, Sweden). The mice were housed under standard conditions. Food and drinking water were available *ad libitum*.

*Experimental design.* The experiment was conducted using nine week-old female BALB/c mice. All treatment groups had comparable body weights of  $20.1 \pm 0.3$  g. Colitis was induced by 2.5% DSS (mol. Wt 40 kD; TdB Consultancy, Uppsala, Sweden), dissolved in purified drinking water, for 19 days. All treatment groups received daily active compound or vehicle from day 13 until the end of the experiment (day 19). Thus, treatment with the compounds studied started first after onset of signs of colitis. This protocol enabled us to monitor potential degree of clinical remission. Effects of rectal administration of antibodies were compared with effects seen after rectal administration of methotrexate, 5-ASA, and oral treatment with azathioprine.

## Treatments

*Antibodies.* Function-blocking monoclonal antibodies Ha1/29 (hamster anti-rat) (Serotec, Oxford, England) and TA-2 (rat anti-mouse) (BD Pharmingen, San Diego, CA, US) directed against the  $\alpha 2$  subunit (CD49b) of the  $\alpha 2\beta 1$

integrin (CD49b/CD29) and the  $\alpha$ 4 subunit (CD49d), respectively, were used. The antibodies were administered rectally through an injection catheter (X-Ray Opaque feeding tube (Vygon, Ecouen, France)). A dose of 20  $\mu$ g antibody in 50  $\mu$ L purified water was administered daily, starting on day 13 after DSS was given and throughout the duration of the experiment.

*Conventional treatments.* Mesalazine (Pentasa<sup>®</sup>) (Apoteket AB, Stockholm, Sweden) 0.06 mg/kg, 0.3 ml/day and Methotrexate (MediGelium AB, Stockholm, Sweden), 100  $\mu$ M, 0.1 ml/day were both administered rectally in the same way as the antibodies. Azathioprine (Imurel<sup>®</sup>, Apoteket AB, Stockholm, Sweden) 10 mg/kg was given in the drinking water.

## Assessments

*Daily assessment of colitis.* Daily assessment of animals included measurement of drinking water volume, body weight and evaluation of blood in feces and diarrhea. When the animals were killed, fecal samples were collected for analysis of blood content by Hemocult IVD (TRIOLAB, Mölndal, Sweden).

*Histopathological analysis.* The removed mice colons were stained with haematoxylin-eosin. The degree of inflammation on microscopic sections of the colon was graded in a blinded fashion by a GI pathologist (JL). A subjective grading system of acute inflammatory activity in four steps was adopted. Normal mucosa was graded as 0, sporadic scattered segmented granulocytes in the lamina propria was defined as grade 1, few granulocytes in smaller foci was defined as grade 2. Larger quantities of granulocytes in lamina propria or if granulocytes were seen in crypts, the inflammation was defined as grade 3. Lymphocytes and plasma cells in the mucosa were not included in the grading system. The surface extension of the mucosal damage was also quantified in the circular microscopic sections of the distal colon and expressed as percentage of the total circular section.

## Statistical analysis

Statistical significance was evaluated employing ANOVA or Student's t-test when appropriate. Values are expressed as mean and standard error of the mean (SEM) unless otherwise stated.  $P < 0.05$  was considered statistically significant.

## Results

### Clinical evaluation of DSS-induced colitis

Clinical evaluation of DSS colitis included daily monitoring of the animals body weight, stool consistency, occult and gross rectal bleeding. All mice that received regular water were negative for clinical signs of colitis throughout the entire experiment. As shown in figure 1, animals that received DSS, substantially lost body weight during the test period. At day 19, when the experiment was ended, DSS-treated animals without treatment had lost  $11.2 \pm 1.1\%$  in body weight compared with healthy controls. Only rectal administration of methotrexate completely prevented weight loss ( $2.7 \pm 1.5\%$  decrease in weight,  $p < 0.05$  vs. DSS alone) whereas anti- $\alpha 2$  and anti- $\alpha 4$  integrin showed a trend of reduced weight loss, however not significant (anti- $\alpha 2$ :  $9.1 \pm 1.0\%$  decrease in weight, vs. DSS alone; anti- $\alpha 4$ :  $9.8 \pm 2.6\%$  decrease in weight, vs. DSS alone). 5-ASA did not prevent weight loss.

Treatment against anti- $\alpha 2$  and anti- $\alpha 4$  integrin were the only treatment that resulted in a reduction of rectal bleeding analyzed by Hemocult IVD as shown in figure 2a. As shown in figure 2b, treatment with integrin antibodies could exclusively also reduce diarrhea vs. DSS alone.

### Histological evaluation of DSS-induced colitis

Histological examination of the distal colon revealed mucosal lesions, edema, crypt damage, and inflammatory infiltrates. Both severity and extension of mucosal damage are displayed in table 1. As displayed in the table 1, methotrexate, azathioprine and integrin-blockade of  $\alpha 2$  could ameliorate both severity and the extension of mucosal damage whereas anti- $\alpha 4$  treatment did not influence the histological pattern compared to DSS-treated animals without treatment, significantly.

Interestingly, in all three treatment groups that received anti- $\alpha 2$  treatment, azathioprine or methotrexate, some animals had a completely undamaged mucosa (expressed as percentage 'responders' in table 1). Most animals with a fully intact mucosa were found in the anti- $\alpha 2$ -treated group.

Colonic shortening is a prominent indicator of IBD. As shown in figure 3, colon length in healthy animals was  $9.5 \pm 0.4$  cm and  $6.0 \pm 0.1$  cm in animals receiving DSS alone (19 days). Only treatment anti- $\alpha 2$  antibody could significantly

prevent colonic shortening as seen in figure 3 ( $p < 0.05$  vs. DSS alone (19 days)). Figure 4 displays representative histological sections of the distal colon of all treatment groups. The most striking histopathological changes in response to DSS were epithelial damage and mucosal crypt destruction. These pathological changes were seen in all treatment groups receiving DSS, however at different levels of severity as previously described in table 1.

## Discussion

In the present study, we investigated the therapeutic potential of local anti-integrin treatment of IBD, and compared the effects with common and well-known drugs used in the treatment of the disease. The DSS model in mice, a well established disease model of chronic colitis, was used. The model is well characterized and involves the activation of both neutrophils and monocytes with minor contribution of lymphocytes in the early phase.

Treatment of animals with therapeutic compounds started first when clinical signs of colitis such as weight reduction and fecal blood were obvious. The therapeutic compounds tested included function blocking monoclonal antibodies against the  $\alpha$  subunit of the  $\alpha 2$  integrin (CD49b) and  $\alpha 4$  integrin (CD49d), rectally administered methotrexate, systemically administered azathioprine and rectally administered 5-ASA. All non-integrin compounds are well established in the clinical treatment of IBD and have well-documented effects on disease modulation. In our comparison of different therapeutic compounds, we found that integrin-blockade, both with antibodies against the  $\alpha 2$  and  $\alpha 4$  integrins, were more effective in suppressing rectal bleeding and diarrhea than other compounds tested. However, anti-integrin treatment was not as effective as locally administered methotrexate in preventing weight loss caused by DSS colitis. The histological evaluation was in favor for anti- $\alpha 2$  integrin treatment and methotrexate. The majority of anti- $\alpha 2$  integrin-treated animals showed no mucosal damage whatsoever. Methotrexate was most effective in reducing the severity of inflammation, as defined by standard histological grading including depth of mucosal damage and infiltration of inflammatory cells. However, only one methotrexate-treated animal showed complete absence of mucosal damage.

In summary, our data show different positive clinical and histological effects of the different treatment regimens. No single compound had superior effect in all parameters studied. Most likely this is due to the different mechanism of action through which the administered compounds act on different parameters of the DSS model.

Whereas anti-integrin treatment is likely to regulate DSS colitis through direct modulation of leukocyte recruitment, both azathioprine and methotrexate intervene with the inflammatory cascade at several, and early levels. Thus, anti-integrin treatment is believed to be more specific in its mode of action. Several compounds directed against cell adhesion molecules have been tested as treatment for Crohn's disease and ulcerative colitis and to date, at least four different cell adhesion molecules have been demonstrated to ameliorate disease activity in experimental models of colitis (7, 12, 22-26).

In this study we present experimental data comparing treatment of DSS experimental colitis with conventional small molecule compounds. Our findings indicate particularly beneficial effects on our DSS model of anti- $\alpha$ 2 treatment and methotrexate. These observations support the concept that integrins, and in particular  $\beta$ 1 integrins, play a major role in both intravascular and extravascular events of leukocyte recruitment in IBD and that anti-integrin regimens may have a therapeutic potential beyond current treatment regimens with 5-ASA, immunomodulators and cytostatic compounds. More comparative analyses of treatment outcome for IBD is however required.

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## Table

**Table 1**

A subjective grading system of acute inflammatory activity in four steps was adopted. Normal mucosa was graded as 0 and defined as responder if the animal had received DSS, sporadic scattered segmented granulocytes in the lamina propria was defined as grade 1, few granulocytes in smaller foci was defined as grade 2. Larger quantities of granulocytes in lamina propria or if granulocytes were seen in crypts, the inflammation was defined as grade 3. The surface extension of the mucosal damage was quantified in the circular microscopic sections of the distal colon and expressed as percentage of the total circular section.

	DSS alone (19 days)	Ha 1/29 (anti- $\alpha$ 2 integrin)	TA-2 (anti- $\alpha$ 4 integrin)	5-ASA	Azathioprine	Methotrexate
Responders,%	0	60	0	0	50	25
Acute inflam., grade 1-3	1.9 $\pm$ 0.3	0.8 $\pm$ 0.5	2.3 $\pm$ 0.1	1.5 $\pm$ 0.3	0.8 $\pm$ 0.5	0.6 $\pm$ 0.2
Damage of the mucosa,%	57 $\pm$ 13	31 $\pm$ 17	63 $\pm$ 10	70 $\pm$ 17	31 $\pm$ 22	19 $\pm$ 14

Data are expressed as mean $\pm$ SEM.

## Legends

**Fig. 1**

Body weight from day 13 compared with healthy controls. All animals received DSS from day 1. Only rectal administration of methotrexate completely prevented weight loss (2.7  $\pm$  1.5% decrease in weight, ( $p < 0.05$  vs. DSS alone) whereas anti- $\alpha$ 2 (Ha 1/29), anti- $\alpha$ 4 integrin (TA-2), and azathioprine showed a trend of reduced weight loss, however not significant. 5-ASA had no effect.

**Fig. 2 a, b**

Inflammatory parameters from day 19, Hemocult IVD was used to analyze blood in feces (a). Diarrhea (b) was scored as no diarrhea (=0) or presence of diarrhea (=1). Note the effects of anti- $\alpha$ 2 antibody (Ha 1/29) anti- $\alpha$ 4 integrin (TA-2) on fecal blood.

**Fig. 3**

Colon length in healthy animals and in DSS-treated animals with different treatments. Box plot with median values. Only treatment with anti- $\alpha$ 2 antibody (Ha 1/29) could prevent colonic shortening ( $p < 0.05$ ).

**Fig. 4**

Haematoxylin-eosin staining showing circular section of the distal colon from healthy controls (A), DSS alone (19 days) (B), anti- $\alpha$ 2 integrin (Ha 1/29) (C), anti- $\alpha$ 4 integrin (TA-2) (D), azathioprine (E), 5-ASA (F), and methotrexate (G). The images are representative for each treatment group.

# Figures

Figure 1

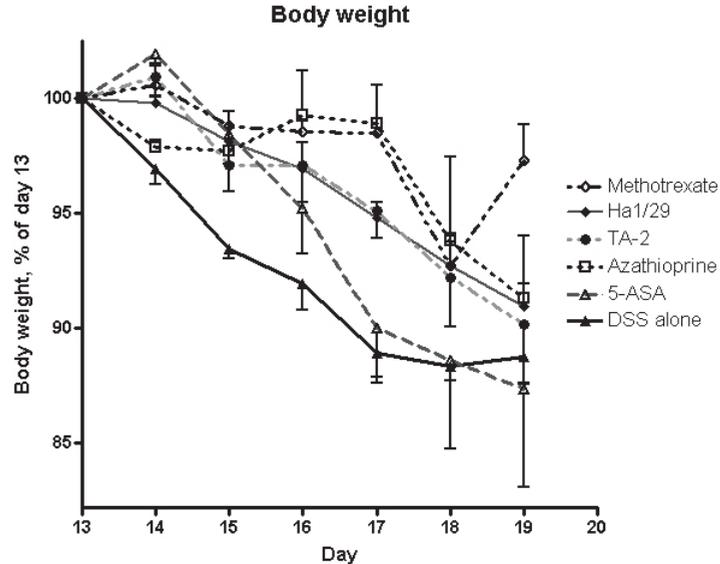




Figure 3

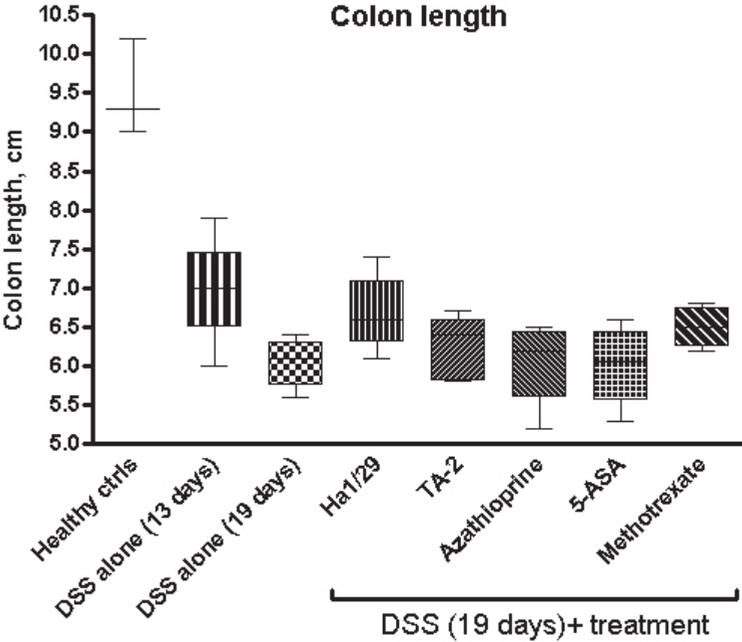


Figure 4

