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Increase in tumor necrosis factor-α production linked to the toxicity of indomethacin for the rat small intestine

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1 The toxic effects of nonsteroidal anti-inflammatory drugs for the lower gastrointestinal tract share certain features with inflammatory processes, suggesting that the release of inflammation cytokines such as TNF-α may damage the intestine.

2 Rats received a s.c. injection of indomethacin. Then, jejunum-ileum was taken up for the quantification of ulcerations, production of TNF-α, nitrites and PGE2 *ex vivo* and activity of calcium-independent NO synthase and myeloperoxydase. Activation of NO metabolism and myeloperoxydase were measured as potential effectors of TNF-α.

3 Jejunum-ileum from rats having received indomethacin (10 mg kg^{-1}) produced TNF-α *ex vivo*. Cytokine production was associated with the onset of macroscopic ulcerations of the small intestine, and preceded nitrite production and tissue activity of myeloperoxidase.

4 Similar intestinal ulcerations and upregulation of TNF-α were obtained with flurbiprofen (30 mg kg^{-1}), chemically unrelated to indomethacin.

5 TNF-α production was proportional to the indomethacin dose (from 3–20 mg kg^{-1}) and correlated with the surface area of ulcerations and nitrite production, 24 h after indomethacin administration.

6 Pretreatment of rats with RO 20-1724, a type-IV phosphodiesterase inhibitor which inhibits TNF-α synthesis, substantially reduced jejunum-ileum ulcerations, TNF-α and nitrite production and tissue enzyme activities.

7 These findings provide evidence that TNF-α is increased in indomethacin-induced intestinal ulcerations and support suggestions that TNF-α is involved at an early stage of nonsteroidal anti-inflammatory drug toxicity for the small intestine.

Keywords: TNF-α; small intestine; nonsteroidal anti-inflammatory drugs; intestinal inflammation; prostaglandins; RO 20-1724; type IV phosphodiesterase inhibitors; nitric oxide; inducible NO synthase; myeloperoxidase

Introduction

Short-term and long-term administration of nonsteroidal anti-inflammatory drugs (NSAID) can cause intestinal damage, including ulcers complicated by perforation and bleeding, strictures and enteropathy with loss of albumin and iron (Bjarnason & Macpherson, 1992; Gargot & Chaussade, 1993). Being frequently asymptomatic and difficult to diagnose, these adverse effects are probably underestimated. Endoscopic studies have indicated that 27% of patients receiving NSAIDs for rheumatoid arthritis have deep jejunum-ileum (JI) ulcerations (Morris *et al*., 1991). Post-mortem studies showed that 8.4% of NSAID users had non specific ulcerations of the small intestine and 4% of long-term NSAID users died as a direct consequence of these ulcers (Allison *et al*., 1992).

Acute and chronic ulcerations of the small intestine can be induced in rats by indomethacin and other NSAIDs (Brodie *et al*., 1970). Up to 80% of the jejunum-ileum is damaged (Weissenborn *et al*., 1985), with transmural ulcers, wall thickening, adhesions, granulomatous inflammation, crypt abscesses and fibrosis (Yamada *et al*., 1993); all these histological features are found in inflammatory bowel disease. An early event in intestinal ulcerations is increased mucosal permeability, which occurs within 24 h after NSAID administration and can be restored by exogenous prostaglandin (PG) administration (Davies *et al*., 1994; Whittle *et al*., 1995). Intestinal microvascular injury can be related to induction of calcium-independent NO synthase (NOS), which began to increase 18 h after indomethacin injection, but the events leading to induction of inducible NOS are not clear (Whittle *et al*., 1995). McCafferty *et al*. (1995) have shown that neutrophils and adhesion molecules play a key role in NSAID-induced gastropathy. In contrast, influx and activation of these cells in the small intestine is more probably a consequence of the inflammation than a key pathogenic process (Yamada *et al*., 1993).

Tumor necrosis factor-α (TNF-α) is a primary cytokine that initiates the inflammatory cascade by inducing secondary cytokines and key enzymes (collagenase, phospholipase A2 and inducible cyclooxygenase and NO synthase). Eicosanoids and nitric oxide (NO) or its metabolites subsequently contribute to signs of inflammation and tissue lesions (Dofferhoff *et al*., 1991; Baggarwal & Natarajan, 1996). TNF-α production is strongly down-regulated by hormones and other soluble mediators. In particular, PG of the E series (PGE) inhibits TNF-α synthesis in a feed-back loop, via increased intracellular concentrations of cAMP (Spengler *et al*., 1989; Ferreri *et al*., 1992). Conversely, inhibition of PG synthesis by NSAIDs can increase TNF-α production *in vitro* and in biological fluids *in vivo* (Utsunomiya *et al*., 1994; Martich *et al*., 1991; Goncalves de Moraes *et al*., 1996).

The small and large bowels are major sites of pathological production of inflammation cytokines, as observed in acute

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and chronic inflammatory bowel diseases (Sartor, 1994). Once the epithelial barrier is broken, cells of the lamina propria are particularly exposed to the detrimental actions of bacteria and bile. Given the intestinal lesions induced by parenteral administration of recombinant TNF-α (Tracey *et al*., 1986; Remick *et al*., 1987), the ability of the intestinal mucosa to produce this cytokine, and the ability of NSAIDs to augment TNF-α production, we investigated the possible involvement of TNF-α in the pathogenesis of small bowel ulcerations induced by NSAIDs. TNF-α production in JI was quantified in rats having received a s.c. injection of indomethacin (10 mg kg^{-1}). Experiments were designed to determine whether TNF-α production (a) was linked in time to the onset and healing of JI ulcerations, (b) was indomethacin dose-dependent (3–20 mg kg^{-1}); and (c) could be blocked, together with the ulcerative process, by rat pretreatment with RO 20-1724, a type 4 phosphodiesterase inhibitor that inhibits TNF production (Verghese *et al*., 1995; Seldon *et al*., 1995). Neutrophil infiltration was assessed in JI in terms of myeloperoxidase activity, and nitrite and calcium-independent NOS activity were measured, as potential effectors of TNF-α. Finally, the ulcerogenic and proinflammatory properties of indomethacin were quantified at the gastric level.

Methods

Animals

Nine-week-old male Wistar rats (Iffa Credo, L'Arbresle, France) received indomethacin subcutaneously, in the interscapular region, in a volume of 5 ml kg^{-1}. The animals were allowed free access to water, were fasted for 16 h before indomethacin administration and were re-fed 5 h later. This protocol was chosen because gastropathy, which develops within a few hours of indomethacin administration, is increased by fasting. JI ulcerations develop later, and are maximal in refeeding animals (Brodie *et al*., 1970; Weissenborn *et al*., 1985; Gargot & Chaussade, 1993). The rats were decapitated (Harvard Apparatus, Small Animal Decapitator) and stomach and JI were removed and gently rinsed with phosphate-buffer saline pH 7.4 (Gibco, Life Technologies, France). The stomachs were opened along the great curvature and the JI was opened along the antimesenteric border. Both tissues were photographed to quantify ulcerations. Samples of the corpus of the stomach (about 200 mg each) were taken for the determination of NOS and MPO activities. Another sample was taken for tissue culture *ex vivo*. Preliminary experiments showed that JI lesions after 10 mg kg^{-1} indomethacin extended to about 50 cm above the cecum. For each analysis, three pieces of JI, 1 cm in length, were excised 10, 20 and 30 cm above the ileo-caecal valve and pooled for enzyme determination (MPO and NOS) and *ex vivo* measurement of TNF-α, nitrite and PGE_2 production.

Indomethacin administration

Indomethacin was dissolved in dimethylsulfoxide (DMSO) and diluted in RPMI 1640 medium (reference R 7509, without antibiotics, Sigma) to a final DMSO concentration of 2%. Control rats received the solvent for indomethacin. Preliminary experiments showed no differences between solvent-treated and untreated rats in terms of TNF-α and nitrite production and NOS activity. In time-course studies, all the rats received 10 mg kg^{-1} indomethacin and four rats were killed at each time point (from 0–96 h). To assess TNF-α production in the digestive tract, four rats each received solvent or 10 mg kg^{-1} indomethacin. Twenty-four hours later 11 portions representative of the digestive tract (the whole esophagus, 1/5 of the stomach, the whole duodenum and 1/4 of the colon) were removed from each rat and cultured as described above. JI was cut into seven pieces 9–10 cm long, and half of each piece was cultured separately. To assess the regional relationship between damage score and TNF-α production in the small intestine, three rats received 20 mg kg^{-1} indomethacin. Twenty-four hours later, the whole small bowel was removed, cut into 10 pieces 6 cm long and each piece was photographed then cultured separately. In the dose-response study, each rat group ($n = 5$) received solvent or 3, 7, 10 or 20 mg kg^{-1} indomethacin. The animals were killed 24 h later, the time of peak JI TNF-α production. The indomethacin dose range covered pharmacological doses and doses toxic for the small intestine (Weissenborn *et al*., 1985). RO 20-1724 was dissolved in 0.5% methylcellulose containing 2% DMSO. Rats received two intraperitoneal injections of RO 20-1724 or solvent, the first 1 h before and the second 8 h after 10 mg/kg^{-1} indomethacin. Rats were killed 24 h later and the stomach and JI (about 200 mg of each) were sampled for histological examination. None of the treated rats died during these different protocols.

Flurbiprofen administration

Flurbiprofen was dissolved in DMSO and diluted in 0.5% methylcellulose to a final DMSO concentration of 5%. It was injected subcutaneously at a dose of 30 mg kg^{-1}, in a volume of 5 ml kg^{-1}. Rats were killed 24 h later to quantify JI ulcerations, and TNF-α production *ex vivo*. Control rats received the solvent.

Gastric and intestinal damage scores

Indomethacin induces gastric linear ulcerations. Damage was scored by measuring the length of ulcerations on 2-fold-enlarged photographs with a curvimeter; the observer was unaware of the treatment. The gastric damage score (expressed in cm) was obtained by adding ulcer lengths (McCafferty *et al*., 1995). JI ulcerations were round, segmental and sometimes confluent. Damage was scored by tracing the outline of ulcerated areas from 2-fold magnified photographic images onto paper and weighting the cut-outs. The JI damage score (expressed in cm^2) corresponds to the sum of ulcerated areas.

Histologic studies

Samples of stomach and small intestine were fixed in Bouin's solution, dehydrated in ethanol and embedded in paraffin. Five-micron sections were stained with hematoxylin-eosin and periodic acid-Schiff according to the standard procedure. Lesions were scored semi-quantitatively as follows: 0 = absent, + = mild, + + = moderate and + + + = severe, after blinded examination of all the slides. The following items were scored individually: number and depth of ulcerative areas; infiltration by inflammatory cells (especially neutrophils) near the ulcerations; infiltration by inflammatory cells in the submucosa; peritonitis; and intravascular thrombi.

NOS activity in tissue samples

Tissue calcium-independent NOS activity, which was assimilated to inducible NOS (iNOS), was estimated by measuring the conversion of L-^{14}C-arginine to L-^{14}C-citrullin as described by Bush *et al*. (1992). Tissue samples (approximately 250 mg)

were homogenized in buffer (pH = 7.4) containing 50 mM Tris-HCl, 1 mM dithiothreitol, 23.4 μM leupeptin, 14.6 μM pepstatin and 1 mM phenylmethylsulfonylfluoride (PMSF). After sonication on ice and centrifugation at 10,000 *g* for 30 min at 4°C, 100 μl of the supernatant was added to a reaction mixture containing 50 mM Tris HCl (pH = 7.4), 1.58 μM L-^{14}C-arginine, 200 μM NADP, 10 μM flavin mononucleotide, 10 μM flavin adenine dinucleotide, 1 mM dithiotreitol, 50 μM tetrahydrobiopterine and 5 mM valine, with 1 mM $CaCl_2$ (total NOS activity) or 1 mM EDTA and 1 mM EGTA (iNOS activity). After 45 min of incubation at 37°C, the enzymatic reaction was terminated by adding cold phosphate buffer (pH = 5.5) containing 3 mM EDTA. L-^{14}C-citrulline was separated by applying the samples to columns containing pre-equilibrated Dowex AG50W-X8, eluting them with water and measuring the amount of radioactivity by means of scintillation counting. Protein content was measured according to Lowry (1951). Enzyme activity is expressed as picomole of citrulline formed per milligram of protein per hour. Specific NOS activity was defined as citrulline formation that was abolished by incubation of the supernatants with L-NMMA (300 μM).

MPO activity in tissue samples

MPO activity was measured as described by Maehly and Chance (1954). MPO was extracted from tissue (frozen at −80°C) by suspending the material in lysis buffer containing 20 mM KH_2PO_4 and 1.4 mM hexadecyltrimethyl ammonium bromide (HTAB) (pH = 6.0), before homogenization on ice with a Polytron homogenizer. Then the homogenate was sonicated for 10 s on ice, frozen at −80°C, thawed, sonicated again for 10 s on ice and centrifuged. The suspension was assayed spectrophotometrically for MPO activity: 500 μl of suspension was combined with 2500 μl of buffer (pH = 6.0) containing 0.16 mM Na_2HPO_4, 18.4 mM KH_2PO_4, 44.8 μM guaiacol and 0.00025% hydrogen peroxide. The kinetics of absorbance at 470 nm was recorded with a spectrophotometer thermostated at 40°C. One unit of MPO activity is defined as that degrading 1 μmole of peroxide per minute. The change in absorbance for each sample was expressed in international units (i.u.) by using a standard curve of horseradish peroxidase established in the same experimental conditions. MPO activity is expressed as milliunits (mu) per gram of total protein, measured according to Lowry (1951).

Culture ex vivo

Gastric and intestinal tissue were carefully washed three times in sterile and apyrogen normal saline, then cultured in 24-well plates (Nuncleon, France) with 1.6 ml of RPMI 1640 medium (Sigma, St Quentin Fallavier, France) without phenol red and supplemented with 100 iu ml^{-1} penicillin/100 μg ml^{-1} streptomycin. Tissues were cultured for 5 h in a humid atmosphere at 37°C with 5% CO_2. Supernatants were then collected and kept at −80°C until the determination of TNF-α, nitrites and PGE_2. Preliminary experiments showed that (a) supernatants were not bacterially contaminated, (b) when tissues were cultured for 1, 3, 5 and 8 h, 90% of TNF-α production occurred within 5 h, which was the time chosen for further experiments, (c) untreated and control rats receiving indomethacin solvent produced less than 25 u TNF-α g^{-1} tissue and 20 nmol nitrites g^{-1} tissue; (d) TNF-α production by indomethacin-treated rat tissues was abolished by incubation at 37°C for 2 h with TN3-19.12, a monoclonal hamster antimouse TNF-α antibody which neutralizes rat TNF-α (Genzyme, Paris, France) (supernatants alone: 113.2 ± 12.7 u ml^{-1} TNF-α; with 5 μg ml^{-1} irrelevant hamster IgG: 110.4 ± 7.9 u ml^{-1}; with 5 μg ml^{-1} antiTNF-α antibody: 0.6 ± 0.1 u ml^{-1}) (mean ± s.e.mean, $n = 3$).

TNF-α assay

The cytotoxic activity of TNF-α in serial dilutions of tissue culture supernatants was quantified on actinomycin D-treated murine L929 fibroblasts as previously described (Chen *et al.*, 1994). The TNF-α concentration (u ml^{-1}) was defined as the reciprocal of the sample dilution that caused half-maximal cell death. Recombinant human TNF-α (provided by Knoll/BASF, Ludwigshafen, Germany) was used for the standard curve. Final concentrations are expressed as u g^{-1} of cultured tissue.

For the time-course study, TNF-α activity was quantified in culture supernatants and also in tissue samples. For the later, JI tissue was homogenized in normal saline supplemented with aprotinin (1 iu ml^{-1}) and 1% fetal calf serum. Centrifugation supernatants were tested for TNF-α activity. A correlation was found between tissular TNF-α and TNF-α production by tissue cultured *ex vivo* ($r = 0.55$, $n = 36$, $P < 0.005$) but the technique with homogenized tissue was less sensitive, with only a 4.5-fold increase 24 h after indomethacin administration. We thus chose TNF-α *ex vivo* production for further experiments.

In the study with RO 20-1724, supernatant TNF was determined by bioassay. Supernatants were also assayed in duplicate for TNF-α protein with a specific ELISA kit (Biosource/Medgenix, Rungis, France) in order to determine whether the decrease in TNF bioactivity is associated with a decrease in the production of TNF-α protein. The minimum detectable dose of ELISA kit is 4 pg ml^{-1} and the intra- and interassay variations are less than 5%, as indicated by the manufacturer.

Nitrite and PGE_2 assay in culture supernatants

Nitrite concentrations were determined in duplicate by using a spectrofluorimetric method with diaminonaphthalene as substrate (Misko *et al.*, 1993). PGE_2 was measured at two dilutions with a specific EIA kit (Stallergènes, Fresnes, France) with a detection limit of 10 ng ml^{-1}.

Drugs

Indomethacin and flurbiprofen were obtained from Sigma Chemical Co (St Quentin Fallavier, France). Monoclonal hamster antiTNF-α antibody was purchased from Genzyme (Paris, France) and RO 20-1724 from RBI (Natick, MA, U.S.A.).

Statistical analyses

The results are expressed as means ± s.e.mean. Statistical analysis was performed with SigmaStat program. The comparison of two means was performed by the Mann–Whitney U test. Results of the time-course study were analysed by Kruskal–Wallis ANOVA with Dunnett's method as *post hoc* test.

Results

Time-course of TNF-α production in JI after indomethacin administration

Macroscopic JI ulcerations induced by 10 mg kg^{-1} indomethacin appeared at the 8th hour and remained increased up

to the 96th hour (Figure 1a). Ulcerations involved 15.5±6.5% of the total mucosal surface area at the 24th hour and 32.2±8.8% at the 48th hour, as measured in the most extensively damaged 10 cm segment of JI. TNF-α production was increased from the 8th hour to the 48th hour ($P<0.05$ *vs* time 0) with a return to baseline values at the 96th hour, when the acute ulcerations began to decrease (Figure 1b).

As expected in the presence of indomethacin (Whittle, 1981), JI PGE2 production was reduced by 75.2±2.8% from the first to the 24th hour, normalizing at the 48th hour (data not shown). Following TNF-α induction, very high nitrite production was found at the 24th hour (Figure 2a, $P<0.05$ *vs* time 0) and coincided with the induction of tissue iNOS, accounting for 54.8±16.3% of total NOS (ANOVA, $P<0.001$). This enzyme activity was not detected earlier, indicating that calcium-dependent NOS accounted for total NOS activity. By contrast, at the 72nd and 96th hours, iNOS accounted for total NOS activity. MPO activity increases significantly from the 24th to the 96th hour, reaching 6.5 times baseline values at the 72nd hour (Figure 2b).

Kinetics of changes at the gastric level

TNF-α did not appear to be strongly involved in gastric ulcerations. The gastric damage score was maximal between the first and the 8th hour after indomethacin administration (ANOVA from 0 to 96 h, $P=0.002$), while increased TNF-α production was only seen at the 24th hour (ANOVA, ns) (not shown). This production was poorly reproducible, contrary to that observed in JI. Moreover, no significant TNF activity (<20 u g^{-1} tissue) was detected in homogenized gastric tissue at any time after indomethacin administration. The time-course of PGE_2 inhibition was similar to that found in the intestine, extending from the first to the 48th hour, with a mean reduction of 61.2±5.3% relative to time 0. In the stomach, neutrophil infiltration (as judged by MPO activity) was not a result of TNF activation, as peak MPO activity occurred at the 8th hour (12.59±4.55 mu g^{-1} protein *versus* 5.10±1.48 mu g^{-1} protein at time 0), i.e. before peak TNF-α production. No significant variations in nitrite production or iNOS activity were found.

On the basis of these results we chose the time of 24 h for subsequent experiments. We focused on the lower digestive tract toxicity of indomethacin, characterized by late ulcerations and maximal TNF-α production at the 24th hour.

Dose-dependent effects of indomethacin on intestinal TNF-α production

A dose of 3 mg kg^{-1} indomethacin did not induce ulcerations (Figure 3a) or TNF-α production in JI (Figure 3b), although it decreased PGE_2 levels by 58% (Figure 3c). Higher doses were associated with a proportional increase in the intestinal damage score and TNF-α production (Figure 3), as well as nitrite production and iNOS activity (regression analysis, $P=0.001$). TNF-α production in JI increased from 3.0±0.7 u g^{-1} in control rats to 151.5±66.4, 419.3±109.5 and 662.7±103.1 u g^{-1} following the administration of 7, 10 and 20 mg kg^{-1} indomethacin, respectively (Figure 3b). Nitrite concentrations in JI were 8.4±2.2 nmol g^{-1} in control

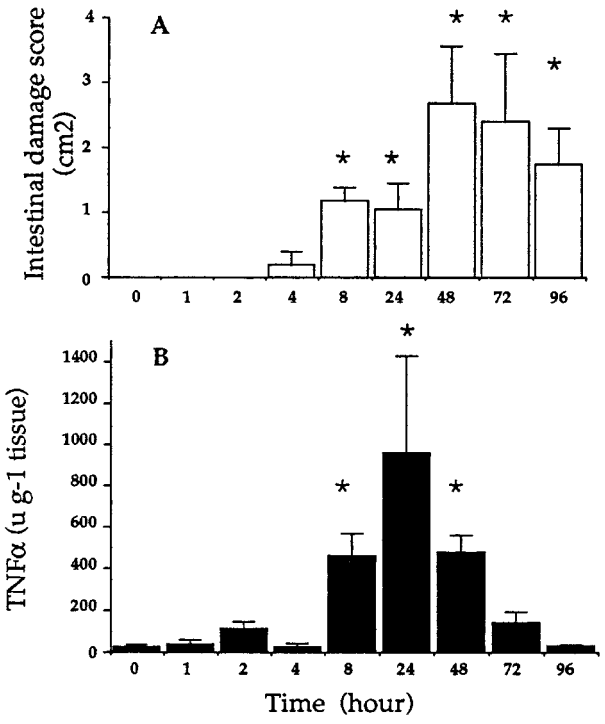

Figure 1 Time course of jejunal-ileal ulcerations (a) and TNF-α (b) production in rats receiving 10 mg kg^{-1} indomethacin by s.c. route, at time 0. Intestinal damage score corresponded to the sum of ulceration area (cm^2). TNF-α was determined in supernatants obtained from JI tissue cultured for 5 h at 37°C. The results are means±s.e.mean ($n=4$ rats per time). ANOVA: (a) ($P<0.001$; (b) $P=0.008$. *$P<0.05$ *vs* value at time 0 (Dunnett's method).

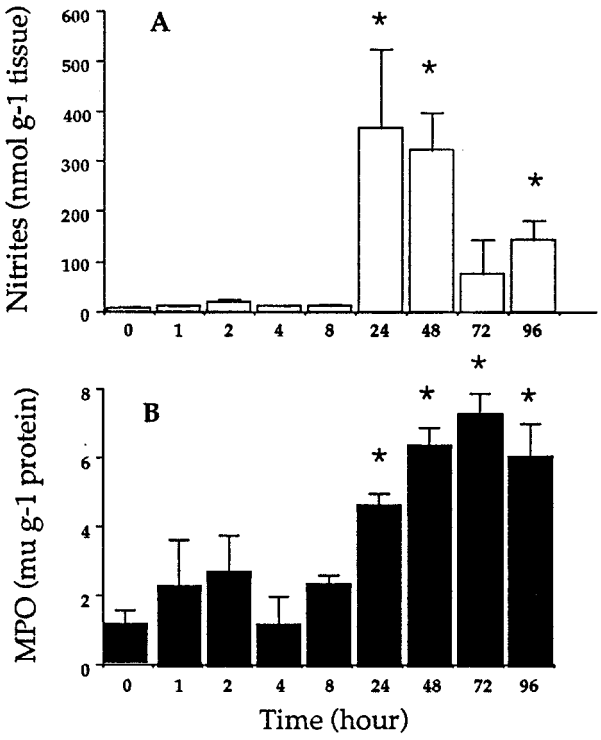

Figure 2 Time course of jejunal-ileal nitrite production (a) and MPO (b) activity in rats receiving 10 mg kg^{-1} indomethacin by s.c. route at time 0. Data were obtained from the same rats than in Figure 1. Nitrites were quantified in supernatants of JI tissue cultured *ex vivo* for 5 h at 37°C. Enzyme activity was determined from homogenized JI tissue as described in Methods. The results are means±s.e.mean ($n=4$). ANOVA: (a) $P=0.01$; (b) $P=0.001$. *$P<0.05$ *vs* value at time 0 (Dunnett's method).

rats and 8.1 ± 1.3 nmol g^{-1} in rats receiving 3 mg kg^{-1} indomethacin. Nitrite concentrations increased to 20.8 ± 7.0, 256.8 ± 95.8 and 929.8 ± 285.6 nmol g^{-1} following the injection of 7, 10 and 20 mg kg^{-1} indomethacin, respectively. Tissue iNOS activity increased from 0.38 ± 0.14 pmol mg^{-1} protein h^{-1} to 1.48 ± 0.43, 2.05 ± 0.32, 2.12 ± 0.40 and 3.58 ± 0.45 pmol mg^{-1} protein h^{-1} following the injection of 3, 7, 10 and 20 mg kg^{-1} indomethacin. TNF-α production by JI correlated strongly with the extent of ulcerations ($r = 0.84$, $n = 25$, $P < 0.001$) and nitrite production ($r = 0.75$, $n = 25$. $P < 0.001$). The inhibition of PGE_2 (Figure 3c) was also, dose-dependent ($P = 0.0017$; regression analysis). Ulcerations and TNF-α release were observed when the decrease in PGE_2 production exceeded 65% (24 h after the injection of indomethacin) (Figure 3a). At the gastric level, TNF-α concentrations were 3.0 ± 3.0, 46.9 ± 19.6 and 152.5 ± 72.2 u g^{-1} with 7, 10 and 20 mg kg^{-1} indomethacin, respectively.

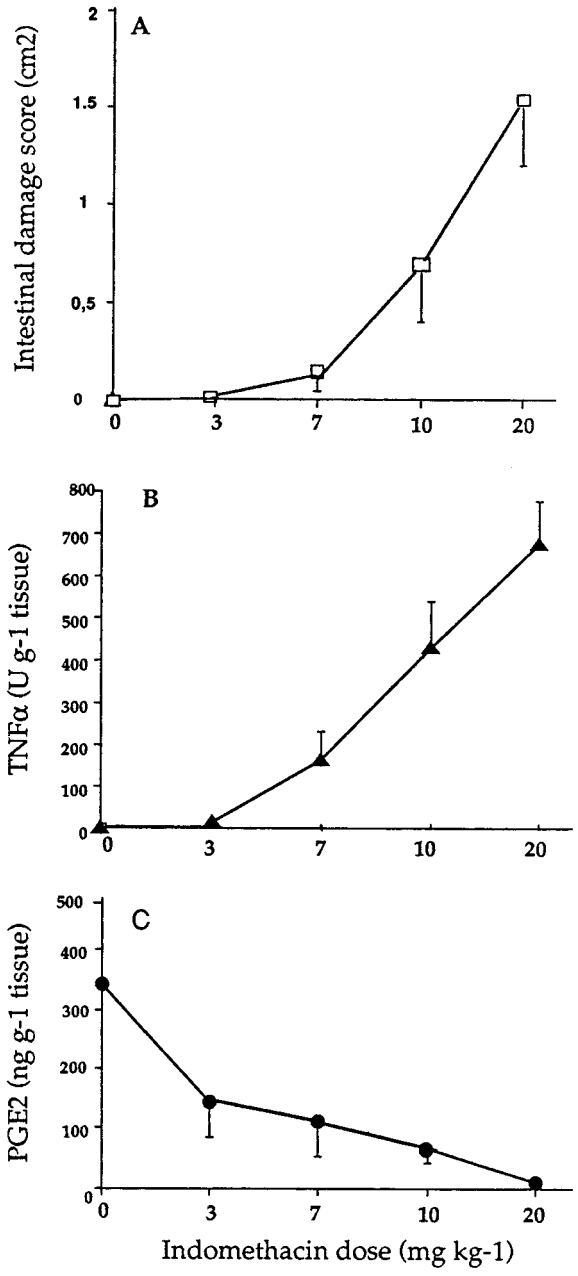

Figure 3 Dose-dependent effects of indomethacin at jejunal-ileal levels. Five rats per group received either solvent, 3, 7, 10 or 20 mg kg^{-1} indomethacin. Twenty-four hours later, rats were sacrificed. (a) Intestinal damage score (sum of ulceration area), (b) TNF-α and (c) PGE2 production in the supernatants of tissue cultured *ex vivo*. The results are the mean ± s.e.mean ($n = 5$ rats per dose).

Production of TNF-α in the gastrointestinal tract 24 h after administration of 10 mg kg^{-1} indomethacin

Control rats showed no macroscopic lesions from the oesophagus to the colon, or significant TNF-α production (Figure 4). In indomethacin-treated rats, healing ulcerations were observed at the gastric level, and one rat had a large duodenal ulcer. JI ulcerations extended along 51.75 ± 3.62 cm ($n = 4$). The aspect of the colon was normal. As shown in Figure 4, the gastrointestinal tract of indomethacin-treated rats produced more TNF-α than that of untreated rats ($P < 0.001$; ANOVA). TNF-α was mainly produced by damaged JI mucosa. TNF-α release by the ulcerated duodenal mucosa reached 142.07 u g^{-1} tissue, while it remained below 25 u g^{-1} tissue in the other three duodenal sections.

Regional relationship between damage score and TNF-α production in the small intestine

In these experiments, rats received 20 mg kg^{-1} indomethacin and all the small bowel was taken up. As shown in Figure 5, damaged sections were associated with TNF-α production and the two parameters were correlated ($r = 0.77$, $n = 30$; $P < 0.001$).

Induction of JI ulcerations and upregulation of TNF-α by flurbiprofen

This experiment was performed to determine whether the effects of indomethacin in JI were shared by other NSAIDs. JI ulcerations induced by 30 mg kg^{-1} flurbiprofen (0.65 ± 0.11 cm^2 *vs* 0 cm^2) extended along 20–30 cm and was associated, as with indomethacin, with very significant

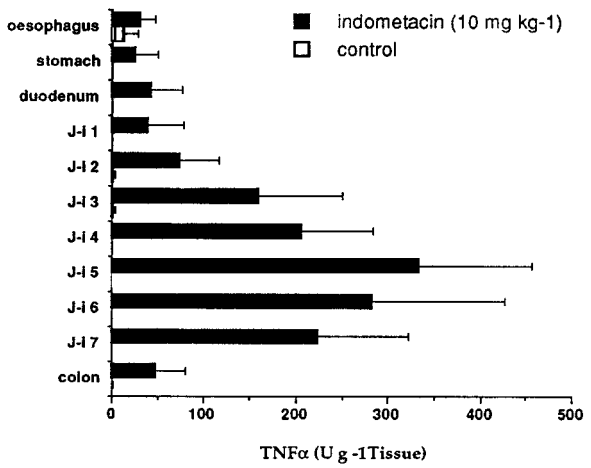

Figure 4 Production of TNF-α along the digestive tract. Four rats received solvent and four rats, 10 mg kg^{-1} indomethacin. Twenty-four hours later they were sacrificed and eleven segments of the digestive tract from the oesophagus to the colon were removed, then washed. They were cultured for 5 h at 37°C *ex vivo*, then TNF-α was quantified in supernatants. The results are means ± s.e.mean ($n = 4$ rats).

production of TNF-α (239.6 ± 31.4 u g^{-1} tissue *vs* 0 u g^{-1}) ($n = 5$ rats per group).

Reduction of TNF-α production and protection from JI ulcerations by RO 20-1724

Pretreatment with 2×20 mg kg^{-1} RO 20-1724 attenuated ulceration area by 78% ($n = 5$; $P = 0.008$) (Table 1). Prevention of JI ulcerations by RO 20-1724 was accompanied by a 88% reduction in TNF-α activity (Table 1). Events occurring after peak TNF-α production (nitrites, iNOS and MPO activation) returned to near-control values in rats treated with both indomethacin and RO 20-1724 (Table 1). JI MPO activity normalized, in keeping with the decrease in infiltrating neutrophils (assessed histologically).

TNF-α protein, determined in the same JI supernatant as TNF-α activity, correlated with TNF-α activity ($r = 0.88$, $n = 15$, $P = 0.001$). TNF-α protein reached 1.94 ± 0.30 ng g^{-1} tissue in rats receiving indomethacin. It was reduced by $76.3 \pm 11.1\%$ in rats treated by RO 20-1724, indicating that the phosphodiesterase inhibitor acted on the amount of TNF-α secreted by the tissue.

Histologically, all the control rats had a normal small bowel mucosa (Figure 6a). Histological evaluation of JI from indomethacin-treated rats revealed numerous ulcerations in these animals, which were often more than 1 cm in length; they were deep, including the mid mucosa, submucosa and underlying tissues (Figure 6b). The ulcer margins were abrupt. Three rats had perforations. When extensive, the ulcers had a necrotic aspect with no cellular reaction, like that of acute ischemic ulcers. Around the mucosal necrosis the adjacent villi were enlarged, with edema and mild inflammatory changes. Lymphatic vessels were distented, and organizing thrombi were observed in small vessels. Adjacent uninvolved mucosa was normal. Moderate amounts of neutrophils infiltrated the submucosa or serosa. Fibrinous peritonitis was observed in all rats and vascular thrombosis was seen in 42% of rats (Figure 6b and Table 2).

In rats receiving indomethacin 2×20 mg kg^{-1} of RO 20-1724, ulcerative lesions of moderate intensity occurred in only 16% of cases, and no rat had severe ulcers. Neutrophil infiltration was mild or moderate around the necrotizing areas. As in the indomethacin-treated rats, flattened villi with lymphatic distensions were observed beneath the ulcerations. Peritonitis occurred in only 30% of rats and vascular thrombosis in only 20% (Figure 6c and Table 2).

In another series of experiments, rat ($n = 5$ per group) were pretreated with 2×10 mg kg^{-1} RO 20-1724. Histologically and at biological level, the protection was similar to that observed with the higher dose of RO 20-1724.

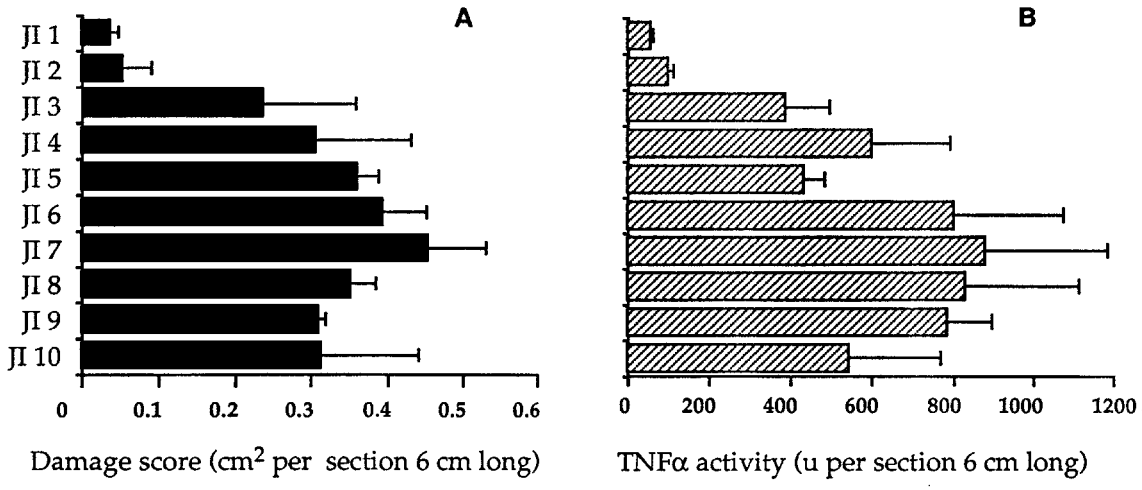

Figure 5 Damage score (a) and production of TNF-α (b) along the small intestine. Three rats received 20 mg kg^{-1} indomethacin. Twenty-four hours later they were sacrificed. All the small intestine was removed, washed, cut in ten segments 6 cm long and photographed. They were cultured for 5 h at 37°C *ex vivo*, then TNF-α was quantified in supernatants. The results are expressed in cm^2 (damage score) and u (TNF activity) per segment. Damage score and TNF-α production were highly correlated ($r = 0.77$, $n = 30$; $P < 0.001$).

Table 1 Effect of RO 20-1724 on damage score, TNF-α and nitrite production, and MPO and iNOS activities, at jejuno-ileal level in indomethacin-treated rats

Treatment	*Damage score* (cm^2)	*TNF activity* (u g^{-1} tissue)	*Nitrites* (nmol g^{-1} tissue)	*iNOS* (pmol mg^{-1} protein h^{-1})	*MPO* (mu g^{-1} protein)
Control	0	44.7 ± 26.0	16.24 ± 3.99	0.45 ± 0.25	3.87 ± 0.38
Indo	1.28 ± 0.21	507.0 ± 55.7	640.7 ± 109.0	7.37 ± 2.95	7.47 ± 1.10
Indo + RO	0.28 ± 0.17	85.8 ± 85.8	46.45 ± 32.29	0.49 ± 0.18	3.31 ± 0.47

Control: rats receiving the solvent; indo: rats receiving 10 mg kg^{-1} indomethacin; indo + RO: rats receiving indomethacin and 2×20 mg kg^{-1} RO 20-1724, 1 h and 8 h after indomethacin. Rats were sacrificed at the 24th h. TNF-α and nitrites were measured in the supernatants of JI tissue cultured for 5 h. Tissular iNOS and MPO activities were determined from homogenized JI tissue as described in Methods. The results are means ± s.e.mean ($n = 5$).

Discussion

The gastric and duodenal damage caused by NSAIDs has long been recognized, but only recently have more distal intestinal disturbances received close attention. The mechanisms by which NSAIDs cause intestinal inflammation are unclear. The gastrointestinal tract is particularly susceptible to the toxic effects of TNF. Its administration causes JI inflammation, with necrosis of villi and endothelial cell damage (Tracey *et al*., 1986; Remick *et al*., 1987). Our study shows that local production of TNF-α probably plays an important role in NSAID-induced intestinal toxicity, together with their action on PG. Indeed, we observed that (a) TNF-α production occurred at the time of macroscopic ulcerations, and before nitrite production and MPO increase; (b) TNF-α release was a function of the indomethacin dose; (c) pretreatment with an anti-TNF drug prevented ulcerations and JI TNF release, and inhibited pathways that can be activated by TNF-α; and (d) flurbiprofen, another NSAID, also induced ulcerations and TNF-α production in JI.

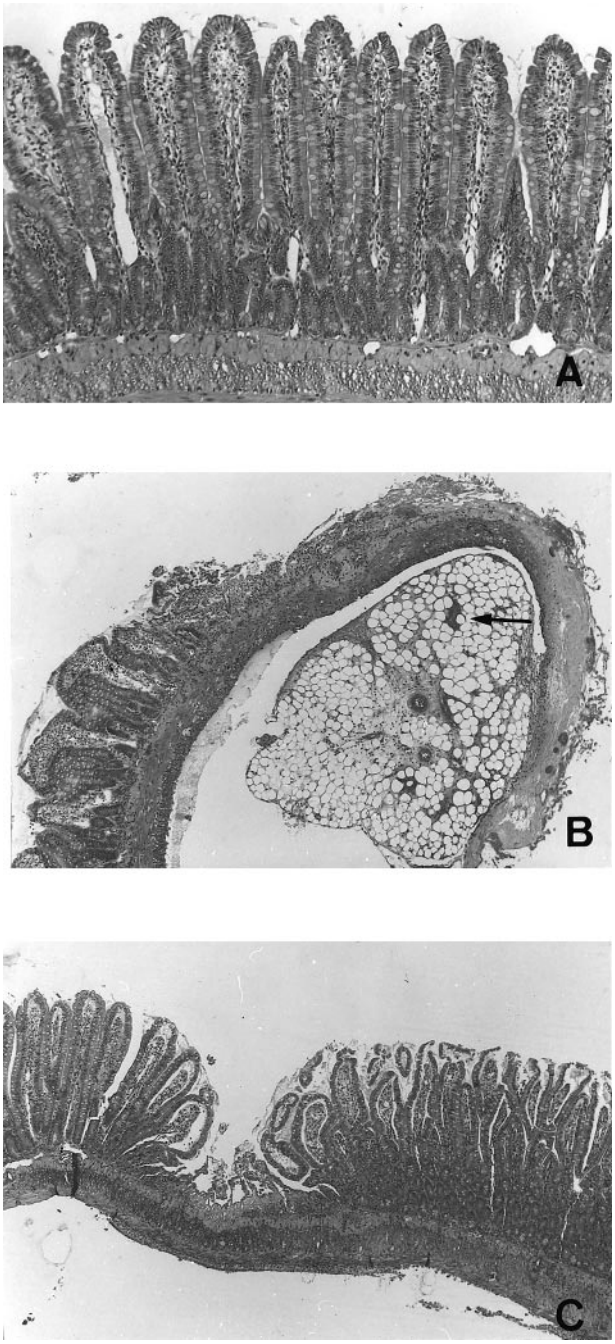

Figure 6 Small bowel from rats receiving (a): solvent (original magnification ×100), (b): 10 mg kg^{-1} indomethacin (original magnification ×40) and (c): indomethacin together with 2×20 mg kg^{-1} RO 20-1724 (original magnification ×40). In indomethacin-treated rat (b) a typical large and deep ulceration is shown with necrosis and perforation. Villi adjacent to the ulcer were enlarged with edema. The arrow shows a thrombus in a small mesenteric arteriole. In rats receiving indomethacin and RO 20-1724, the normal aspect seen macroscopically was confirmed histologically. When ulcer occurred, as shown in (c), it was moderate and reached the submucosa. Ulcer margins were abrupt and some adjacent villi were enlarged.

A negative feed-back loop between PGE_2 and TNF was rapidly recognized as a physiological means of limiting the intensity and duration of TNF effects on its various targets, making TNF a short-lived 'alarm' cytokine (Spengler *et al*., 1989; Ferreri *et al*., 1992). Conversely, NSAID potentiation of TNF production by stimulated macrophages has been observed in various model systems (Goncalves de Moraes *et al*., 1996; Utsunomiya *et al*., 1994) and in humans receiving low doses of endotoxin (Martich *et al*., 1991). The pathophysiological significance of the NSAID-induced increase in TNF production has rarely been evoked. Goncalves de Moraes *et al*. (1996) reported that it was associated with increased neutrophil influx to the inflammatory site. Our results highlight the paradox that these anti-inflammatory drugs have proinflammatory properties at the intestinal level by potently inducing TNF. Contrary to authors using other model systems, we observed that TNF was increased without exogenous stimulation by, for example, lipopolysaccharide or carrageenan. Bacterial endotoxins or superantigens in the intestinal lumen could provide the first stimulus, as germ-free rats are protected from NSAID-induced JI ulcerations (Robert & Asano, 1977). NSAIDs can reactivate or aggravate Crohn's disease (Bjarnasson *et al*., 1992; Kaufmann & Taubin, 1987), whereas these patients respond to anti-TNF antibody (Van Dullemen *et al*., 1995). Our results obtained in rats indicate that induction of JI TNF-α production by NSAIDs is one mechanism by which these drugs could be detrimental in Crohn's disease.

The results with various doses of indomethacin indicated that TNF-α production was detected only with doses higher than 3 mg kg^{-1}, when PGE_2 production was reduced by more than 65% (24 h after NSAID administration). This suggests that partial reduction of PGE_2 is not sufficient to increase TNF-α from intestinal tissue. This can be explained by the fact that low levels of PGE_2 (0.01 μM or less) inhibit TNF-α production from stimulated cells (Kunkel *et al*., 1986; Strassmann *et al*., 1994). Then, 7, 10 and 20 mg kg^{-1} indomethacin reduced PGE_2 levels less rapidly than they increased TNF-α levels. One possibility is that PGE_2 concentrations, measured once ulcerations were induced, do not only represent COX inhibition by indomethacin. The phenomenon is probably more complex, associating a part of induction of PG production via inducible COX, in response to TNF-α. A similar pattern of response was observed in the model of carrageenin-induced pleurisy (Utsunomiya *et al*., 1994).

We found that induction of intestinal TNF production by NSAIDs was followed by activation of two important pathways in the inflammation cascade, which could contribute to JI lesions: the neutrophil activation pathway (MPO activity) and NO metabolism (increased tissue iNOS activity and nitrite production). TNF induces the synthesis of adhesion molecules and chemokines such as IL8, that lead to local recruitment and activation of neutrophils. We found that maximal MPO

Table 2 Effect of RO 20-1724 on histological jejunal-ileal score in indomethacin-treated rats

Treatment	*Ulcerations*	*Neutrophils*	*% of rats Submu. infiltr.*	*Peritonitis*	*Thrombi*
Control	0	0	0	0	0
Indo	moderate: 58 severe: 20	100	78	100	42
Indo + RO	moderate: 12 severe: 0	50	40	30	20

Control: rats receiving the solvent; indo: rats receiving 10 mg kg^{-1} indomethacin indo + RO: rats receiving indomethacin and 2×20 mg kg^{-1} RO 20-1724, 1 h before and 8 h after indomethacin. Rats were sacrificed at the 24th h. JI tissue was paraffin embedded, treated as described in Methods. The results are expressed in percent of total rat number ($n = 5$ rats per group). Submu. infil., submucosa infiltration; Thrombi, intravascular thrombi.

activity occurred 24–48 h after the peak of TNF production, i.e. 48–72 h after indomethacin administration. This coincided with massive neutrophil influx at the 48th hour, observed in immunostained small intestine (Nygard *et al*., 1994). This confirms that neutrophils do not play a key role in the induction of intestinal ulcerations (Yamada *et al*., 1993) but could participate in the aggravation of intestinal lesions.

NO participates in numerous physiological and pathological functions in the gastrointestinal tract (Stark & Szurszewski, 1992). Epithelial intestinal cells can generate NO, and the modulation of NO generation is among the mechanisms responsible for the diverse effects of cytokines, mitogens and bacterial products on the function and integrity of the intestinal mucosa. TNF-α is one of the cytokines capable of inducing iNOS in various cells. Our results showed a time-dependent induction of TNF-α, iNOS activity and large amounts of nitrites in JI of indomethacin-treated rats, suggesting a role of NO in NSAID-induced intestinal injury via the upregulation of TNF-α. Moreover, nitrites were detected only after indomethacin doses that induced macroscopic ulcerations (from 7 mg kg^{-1}). Nitrite concentrations correlated with the intestinal damage score and TNF-α production induced by indomethacin, in dose-dependent fashion. These findings support the view that iNOS induction may be an important local mediator of intestinal damage as previously suggested (Whittle *et al*., 1995). The detrimental properties of NO or its metabolites on the digestive tract have been demonstrated by the protective effects of specific inhibitors on experimental ileitis (Miller *et al*., 1993). We found that calcium-independent NOS activity and nitrite production increased more than 8 h after s.c. administration of indomethacin. Similar late iNOS induction, associated with microvascular leakage, was observed by Whittle *et al*. (1995), whereas JI TNF production clearly increased at an earlier stage (Figure 1), suggesting that iNOS induction is secondary to the TNF elevation.

We found that RO 20-1724, a type 4 PDE inhibitor, reduced TNF-α production and JI ulcerations induced by indomethacin. RO 20-1724 also normalized MPO and iNOS enzyme activities and abrogated JI nitrite production, suggesting a causal relationship between TNF and the other mediators. PDE type 4 is the predominant isozyme present in inflammatory cells and is selectively inhibited by RO 20-1724 and rolipram (Palfreyman, 1995). These drugs exert their anti-inflammatory effects by elevating cAMP, and thereby inhibiting leukocyte functions. The main target of PDE 4 inhibitors is TNF. Its synthesis by monocytes-macrophages is potently inhibited, whereas contradictory results have been obtained for IL1 (Verghese *et al*., 1995; Semmler *et al*., 1993). Our results with RO 20-1724 can hardly be explained by an inhibitory action on iNOS expression and/or activity, because cyclic AMP analogues and specific inhibitor of PDE 4 induce nitric oxide synthesis in peritoneal macrophages (Alonso *et al*., 1995; Jung *et al*., 1997).

Type 4 PDE inhibitors, by increasing cAMP levels, can also down-regulate neutrophil functions. Our results with RO 20-1724 cannot be explained by a primary action on neutrophils, because this cell type is involved late in our experimental model in terms of cell influx (Nygard *et al*., 1994) and enzyme activities (Yamada *et al*., 1993; our results). Moreover, depletion of circulating neutrophils has no effect on intestinal inflammation induced by indomethacin (Yamada *et al*., 1993), contrary to what occurs in the stomach.

PDE 4-selective inhibitors can also attenuate an inflammatory T cell response and down-regulate Th1-type cytokines IL2 and interferon γ (Ganter *et al*., 1997; Giembycz *et al*., 1996). There is no reported evidence that the acute intestinal inflammation induced by NSAID is of lymphocytic origin and TNF-α remains a pivotal mediator, produced by macrophages under the stimulation of Th1 cytokine, during an inflammatory T cell response.

In consequence, although type 4 PDE inhibitors do not act as selectively as antiTNF antibody, our results support the hypothesis that RO 20-1724 reduces intestinal ulceration by inhibiting TNF. However, definite involvement of TNF-α will be established only with the use of antiTNF-α antibody.

Inhibition of intestinal TNF-α production by RO 20-1724 shows that the intestine is another tissue sensitive to PDE 4 inhibitors *in vivo*. Previous studies indicated that RO 20-1724 and rolipram were both active on experimental acute encephalomyelitis (Sommer *et al*., 1995), fulminant liver failure (Gantner *et al*., 1997) and carrageenan-induced paw edema (Sekut *et al*, 1995), an action linked to decreased TNF-α production. Our results suggest that this new pharmacological class could be of interest in inflammatory bowel diseases where inflammatory cytokines play a predominant role (Sartor, 1994). Interestingly, PGE_2 (Strassmann *et al*., 1994) and PDE 4 inhibitors (Kambayashi *et al*., 1995) both inhibit TNF-α release by macrophages, at least partly by augmenting IL-10 production. IL-10 is an immunoregulatory and anti-inflammatory cytokine that inhibits macrophage functions. Mononuclear cells of the lamin propria have the potential to express IL-10 and to respond to it, and mice lacking the gene encoding IL-10 develop inflammatory bowel disease (Kühn *et al*., 1993). Thus, the NSAID effect on JI TNF-α production might conceivably involve negative regulation of IL-10.

We found no significant early production of TNF-α by the gastric mucosa, after indomethacin or flurbiprofen administration. The role of TNF in the gastric ulcerations has been suggested (Santucci *et al*., 1995; Appleyard *et al*., 1996) but to our knowledge, the presence of this cytokine (protein, activity or mRNA) has not been reported in the gastric mucosa.

However, in these studies, the administration of antiTNF compounds, pentoxifylline (a non specific phosphodiesterase inhibitor), antiTNF antibody (Appleyard *et al*., 1996) and RO 20-1724 (our results, not shown) protected from indomethacin-induced gastric ulcerations. We cannot rule out that TNF-α will be transiently involved at gastric level but is barely detectable in the gastric mucosa for reasons that remain to be determined.

The results of the present study suggest the involvement of TNF-α in the mechanism leading to JI ulcerations induced by indomethacin in the rat. If similar TNF induction occurs in human intestine, then antiTNF drugs and more particularly type IV-phosphodiesterase inhibitors, may have potential interest to treat intestinal damage associated with the use of NSAIDs.

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