Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process

JOHN L. WALLACE, CATHERINE M. KEENAN, AND D. NEIL GRANGER
Gastrointestinal Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada; and Department of Physiology and Biophysics, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Wallace, John L., Catherine M. Keenan, and D. Neil Granger. Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. Am. J. Physiol. 259 (Gastrointest. Liver Physiol. 22): G462-G467, 1990.—The hypothesis that neutrophils play an important role in the pathogenesis of gastric ulceration induced by nonsteroidal anti-inflammatory drugs (NSAIDs) was tested in rats. Rats made neutropenic by prior treatment with an antibody to rat neutrophils raised in goat were found to be significantly more resistant to the gastric-damaging actions of indomethacin or naproxen than were control rats or rats pretreated with normal goat serum. The reduction of damage in neutropenic rats was not due to effects of the anti-neutrophil serum on either gastric acid secretion or the ability of indomethacin or naproxen to inhibit prostaglandin synthesis. Gastric cyclooxygenase activity was inhibited by >95% in both normal and neutropenic rats that received indomethacin or naproxen. Reduction of circulating neutrophil numbers by treating rats with methotrexate also resulted in a significant reduction in the susceptibility to gastric damage induced by indomethacin. Since activation of circulating neutrophils appeared to be important in the development of gastric erosions after administration of indomethacin, we investigated the possibility that leukotrienes (LTs) and platelet-activating factor (PAF) might be involved in the pathogenesis of indomethacin-induced ulceration. Changes in gastric LT synthesis were not observed after indomethacin administration. Pretreatment with either an LTD4 antagonist or a PAF antagonist was without significant effect on the extent of gastric damage induced by indomethacin. These results suggest an important role for neutrophils in the pathogenesis of NSAID-induced gastric ulceration. Neutrophils may be important in the vascular injury that occurs early after administration of these compounds.

THE GASTROINTESTINAL IRRITANT properties of nonsteroidal anti-inflammatory drugs (NSAIDs) continue to be the major impediment to their use in the treatment of inflammatory diseases such as rheumatoid arthritis. A recent study (6) demonstrated that patients with rheumatoid arthritis taking NSAIDs are six times more likely to develop gastrointestinal complications than patients not taking these agents. In another recent study, Graham et al. (6) reported that hemorrhagic lesions of the gastric mucosa occurred in >20% of osteoarthritis patients taking ibuprofen, piroxicam, and naproxen. Despite extensive research, the pathogenesis of NSAID-induced damage to the gastric mucosa is still not fully understood. It is generally accepted that inhibition of prostaglandin synthesis is an important component of the ulcerogenic mechanism. However, Ligumsky et al. (14) demonstrated that gastric prostaglandin synthesis in rats could be inhibited by up to 95% without the development of hemorrhagic erosions, suggesting that inhibition of prostaglandin synthesis was unlikely to be the sole mechanism responsible for the ulceration. Furthermore, since the precise physiological role of prostaglandins in gastric mucosal defense is still not completely understood, it is not clear how inhibition of prostaglandin synthesis predisposes the mucosa to injury.

In recent years, it has become increasingly clear that neutrophils and neutrophil-derived factors play an important role in several forms of experimental gastrointestinal ulceration and inflammation. For example, neutrophils have been shown to play an important role in the gastrointestinal damage associated with hemorrhagic shock and ischemia-reperfusion (15-17, 20, 21). Itoh and Guth (10) demonstrated that the gastric damage induced by hemorrhagic shock could be significantly attenuated by free radical scavengers and inhibitors of oxygen radical generation. More recently, Kvietys et al. (13) demonstrated that neutrophils contribute significantly to the gastric damage induced by 20% ethanol. Wallace and Whittle (28) have suggested previously that neutrophils might contribute to the gastric ulceration induced by platelet-activating factor (PAF). This hypothesis was supported by Etienne et al. (4), who demonstrated that neutrophenic rats were significantly more resistant to the gastric damaging actions of PAF than were normal rats. Thus it is possible that neutrophil activation may be a feature common to many experimental models of gastric and intestinal ulceration.

To our knowledge, no study to date has examined the role of the neutrophil in the damage induced in the stomach by NSAIDs. There is, however, some experimental evidence suggestive of such a role. Kitahora and Guth (12) demonstrated that the application of aspirin to the rat stomach resulted in the appearance in the mucosal microcirculation of white thrombi followed by a slowing of mucosal blood flow. Hemorrhagic erosions subsequently formed in these regions. These observa-
tions suggested that neutrophil margination and activation might be occurring during this period and that it might contribute to the subsequent development of hemorrhagic erosions. In the present study, we assessed the effects of neutropenia on the susceptibility to NSAID-induced damage. We also examined the role of two groups of mediators, leukotrienes and PAF, that have been reported to be capable of activating neutrophils in the development of NSAID-induced gastric erosions.

METHODS

Male Wistar rats weighing 200–225 g were deprived of food, but not water, for 18–22 h before an experiment. Gastric hemorrhagic damage was induced by oral administration of either indomethacin or naproxen. Both NSAIDs were dissolved in 5% sodium bicarbonate. Indomethacin was given at a dose of 20 mg/kg, whereas naproxen was given at a dose of 40 mg/kg. In the control groups, the rats received an equivalent volume (0.1 ml/100 g body wt) of the vehicle. In most experiments, damage was assessed 3 h after administration of indomethacin or naproxen. The damage was scored by an observer unaware of the treatment using a system described previously (27) that takes into consideration both the number and size of the lesions. We have previously shown that this estimation of damage correlates well with histological assessment of the damage produced by indomethacin (27).

Time course study of indomethacin-induced gastric damage. Indomethacin was administered orally (20 mg/kg) to four groups of four rats each, while a fifth group of rats (n = 4) received the vehicle. The groups of indomethacin-treated rats were killed at one of the following times after its administration: 15, 30, 60, or 120 min. The control rats were killed 30 min after administration of the vehicle. Gastric damage was scored, and samples of the corpus region of the stomach were excised of mediators, leukotrienes and PAF, that have been reported to be capable of activating neutrophils in the development of NSAID-induced hemorrhagic erosions. The antibody to LTB4 cross-reacts with PGE2 at 5%, PGF2α at 0.3%, and at <0.01% with the other major cyclooxygenase products. The antibody to LTB4 cross-reacts with the other lipoxygenase and cyclooxygenase products at <0.4%.

Additional experiments were performed in which staining of damaged vascular endothelium with Monastral Blue was determined at various times after administration of indomethacin. This marker has been used previously to detect changes in vascular permeability after oral administration of ethanol (22). Rats were given indomethacin (20 mg/kg) orally and were killed 15, 60, or 120 min later (n = 4 per group). Five minutes before the rats were killed, Monastral Blue (9%; 0.1 ml/100 g body wt) was injected intravenously via a tail vein. After the rat was killed, the stomach was rapidly excised, opened by an incision along the greater curvature, and pinned out on a wax platform. The stomach was then immersed in 10% Formalin for 24 h followed by immersion in glycerol for 24 h. The stomach was examined under a light microscope, and the dorsal and ventral sides of the mucosal surface were photographed on color transparency film. The transparencies were coded then projected onto an opaque digitizing tablet attached to a microprocessor. Both the total area of glandular mucosa and the area in which staining with Monastral Blue was evident were determined planimetrically using the digitizing tablet. The total surface area of any hemorrhagic erosions was also determined. The final magnification of the mucosa when planimetry was performed was ×50.

Effects of neutropenia induced by anti-neutrophil serum. Rats were made neutropenic by an intraperitoneal injection of anti-neutrophil serum (ANS) raised in goat against circulating rat neutrophils (21). The rats in the test group received 0.75 ml of the ANS intraperitoneally 12 h before oral administration of indomethacin or naproxen. This volume of ANS was used because it has previously been shown to reduce circulating neutrophil numbers without significantly altering circulating lymphocyte or erythrocyte numbers (21). Control rats received a similar volume of either normal saline or normal goat serum intraperitoneally at the same time. The normal goat serum was diluted in 0.9% saline so that it had the same concentration of protein [determined by the Bradford technique (2)] as the ANS. A blood sample was taken from a tail vein of each rat before administration of ANS, saline, or normal goat serum and immediately before administration of the NSAID. The sample was smeared on a glass slide and stained with a modified Wright's stain (Leukostain; Fisher Scientific). The slides were coded to avoid observer bias then examined under a ×100 objective of a light microscope. The number of neutrophils in 50 fields of view, selected by Vernier coordinates, were counted. Gastric damage induced by the NSAIDs was assessed 3 h after NSAID administration by an observer unaware of the treatment.

An additional group of four rats received indomethacin 12 h after intraperitoneal administration of ANS, as above. These rats were killed 15 min after indomethacin administration, but were given Monastral Blue 5 min before being killed, and the extent of vascular staining with Monastral Blue was determined as described above.

Since gastric damage induced by NSAIDs appears to be a process dependent on the presence of luminal acid, experiments were performed to determine the effects of ANS on gastric acid secretion. Rats pretreated with ANS 12 h earlier (n = 4) and control rats (untreated, n = 4) were anesthetized with pentobarbital sodium (60 mg/kg ip). A laparotomy was performed, and the pylorus was ligated. The rat was then left for 3 h, with body temperature maintained at 37°C through the use of heat lamps. Pedal reflexes were periodically monitored, and when necessary additional pentobarbital sodium was administered. At the end of the 3-h period the esophagus was clamped and the stomach was excised with care taken to preserve the luminal contents of the stomach. The vol-
of the luminal fluid was then diluted in 20 ml of distilled water, and the concentration of acid was determined by titration to pH 7.0 with 0.01 M NaOH using an automated titration apparatus (Metrohm). The total amount of titratable acid present in the sample of gastric juice was then calculated.

Effects of reduction of circulating neutrophil numbers by methotrexate. Groups of five rats were treated with metotrexate using a dosing regimen described previously (9). The rats received methotrexate (2.5 mg·kg⁻¹·day⁻¹ ip) or the vehicle (normal saline) each day for 3 days. Blood samples were taken from a tail vein for estimation of circulating neutrophil numbers (as above) on the day after the final dose of methotrexate or saline and each day thereafter. On the third and fifth days after methotrexate administration, the susceptibility to indo- methacin-induced gastric damage was assessed. A blood sample was taken from a tail vein, then indomethacin was administered (20 mg/kg ip). The rats were killed 3 h later for assessment of gastric damage.

Effects of a PAF antagonist. Groups of five rats each were given the PAF antagonist WEB 2086 (3 or 20 mg/kg) (8) or the vehicle (0.9% saline) intraperitoneally 15 min before oral administration of indomethacin (20 mg/kg) and again 90 min later. The rats were killed 3 h after indomethacin administration, and gastric damage was assessed. These doses of WEB 2086 were selected because in pilot experiments we found that they were effective as inhibitors of PAF-induced hemoconcentration in rats. Briefly, PAF was infused intravenously (100 ng·kg⁻¹·min⁻¹) into anesthetized rats for a period of 10 min. The rats were pretreated with WEB 2086 (3 or 20 mg/kg ip) or the vehicle 15 min before the infusion of PAF (n = 5 per group). Blood samples for hematocrit determination were taken before and 30 min after completion of the PAF infusion. In control rats, PAF infusion caused an increase in hematocrit of 39 ± 6%. Pretreatment with WEB 2086 caused a 61 and 82% inhibition of the PAF-induced changes in hematocrit at doses of 3 and 20 mg/kg, respectively.

Effects of a leukotriene D₄ receptor antagonist. Groups of five rats each were given the LTD₄ antagonist MK-571 (3 mg/kg) (11) or the vehicle (0.9% saline) intraperitoneally 15 min before oral administration of indomethacin. The rats were killed 3 h later, and the extent of gastric damage was assessed. This dose of MK-571 was selected because we have previously found that it completely suppressed LTD₄-induced vasoconstriction in the rat stomach (25).

Statistical analysis. All data are expressed as means ± SE. Groups of data were compared using Student's t test for unpaired data or with an analysis of variance (ANOVA) followed by a Duncan's multiple range test. For the comparison of hemorrhagic damage area to area of Monastral Blue leakage, a paired t test was used. The particular statistical analysis used in each experiment is indicated in the figure legends. An associated probability of 5% or less was considered significant.

Materials. WEB 2086 was obtained from Boehringer (Ingelheim, FRG). The antisera to LTD₄, the unlabeled LTB₄, and the MK-571 were obtained from Merck-Frosst (Pointe Claire, Canada). Indomethacin, naproxen, and Monastral Blue were obtained from Sigma Chemical (St. Louis, MO). The antiserum to 6-keto-PGF₁α and the unlabeled 6-keto-PGF₁α were obtained from Cayman Chemical (Ann Arbor, MI). Labeled LTB₄ and 6-keto-PGF₁α were obtained from Amersham (Oakville, Canada). All other reagents were purchased from Fisher Scientific (Edmonton, Canada).

RESULTS

Time course study of indomethacin-induced gastric damage. Within 15 min of oral administration of indomethacin, gastric cyclooxygenase activity was significantly inhibited. In control rats, the mean level of synthesis of 6-keto-PGF₁α was ~1,200 pg/mg tissue, whereas in the rats killed 15 min after treatment with indomethacin the mean level of synthesis of this prostanoïd was <50 pg/mg (>95% reduction; P < 0.05). Gastric synthesis of 6-keto-PGF₁α was also significantly depressed in each of the other groups of indomethacin-treated rats (up to 3 h postindomethacin).

In contrast to 6-keto-PGF₁α, gastric synthesis of LTB₄ was not significantly affected by indomethacin at any of the times studied (Fig. 1).

The severity of gastric hemorrhagic erosions increased with time after indomethacin administration (Fig. 1). The erosions were invariably linear and confined to the corpus region. They were frequently located on the crests of rugal folds.

Staining of the vascular endothelium with Monastral Blue was not observed in any of the four control rats that were treated with the vehicle for indomethacin. However, as early as 15 min after indomethacin administration significant Monastral Blue staining was detected (Fig. 2). With time after indomethacin administration, there were progressively fewer regions in which Monastral Blue staining was evident in the absence of hemorrhagic necrosis. Examination of transverse sec-

![Figure 1](image-url) **FIG. 1.** Effects of indomethacin administration (20 mg/kg po) on gastric leukotriene B₄ (LTB₄) synthesis and on gastric damage score. Groups of 4 rats each were killed at various times after indomethacin administration. Control rats (time 0) received the vehicle for indomethacin. While the severity of gastric damage increased with time after indomethacin administration (significantly greater than controls at minute 120), gastric LTB₄ synthesis was not significantly different from that in control rats at any of the time points studied (ANOVA and Duncan's multiple range test).
Neutrophils and NSAID-induced ulcer

Monastral Blue Leakage
Hemorrhagic Erosions

FIG. 2. Effects of indomethacin (20 mg/kg po) on permeability of the gastric microcirculation to Monastral Blue. Groups of 4 rats each were killed at various times after administration of indomethacin or the vehicle (controls). Monastral Blue was administered intravenously 5 min before rats were killed. Areas of Monastral Blue leakage and of hemorrhagic damage were determined planimetrically and are expressed as percent of total glandular mucosa. * Significant difference between the area of Monastral Blue leakage and the area of hemorrhagic erosions (P < 0.05; Student's t test).

FIG. 3. Effects of neutropenia induced by administration of anti-neutrophil serum on the susceptibility to gastric damage induced by oral administration of indomethacin or naproxen. Control rats were not treated with anti-neutrophil serum. An additional control group received normal goat serum before administration of the indomethacin or naproxen. * Significant differences from control group (P < 0.01; Student's t test). Each group consisted of 6-12 rats.

 Pretreatment with ANS 12 h before administration of the NSAID resulted in a decrease in circulating neutrophil numbers to <10% of control levels (P < 0.001; control level of 11.5 ± 1.5 neutrophils/50 fields of view). This state of neutropenia was also evident when neutrophil counts were performed 3 h after administration of the NSAIDs. The neutropenic rats were significantly more resistant to the damaging actions of indomethacin or naproxen (Fig. 3). Treatment with normal goat serum did not significantly affect circulating neutrophil numbers nor did it significantly affect the susceptibility to gastric damage induced by indomethacin or naproxen.

 Treatment with ANS did not appear to interfere with the ability of indomethacin to inhibit gastric cyclooxygenase. As shown in Fig. 4, indomethacin significantly (P < 0.01) reduced gastric 6-keto-PGF₁α synthesis regardless of whether or not the rats had been pretreated with ANS. Similarly, ANS did not interfere with the inhibition of gastric prostaglandin synthesis by naproxen (untreated controls, 1,089 ± 309 pg/mg; naproxen alone, 31 ± 16 pg/mg; naproxen + ANS, 43 ± 18 pg/mg).

 In neutropenic rats killed 15 min after indomethacin administration, increased staining with Monastral Blue was not observed, whereas ~2% of the glandular mucosa showed staining with Monastral Blue in the control rats receiving indomethacin.

 It is unlikely that the reduction of NSAID-induced gastric damage in neutropenic rats could be attributable to effects on gastric acid secretion. In experiments in which the pylorus was ligated and the gastric secretions were collected 3 h later, treatment with ANS did not significantly affect the volume (2.1 ± 1.1 vs. 1.3 ± 0.6 ml in controls) or the acid concentration (158.0 ± 98.2 vs. 136.9 ± 87.3 μeq/ml in controls) of the gastric juice. The total acid output during the 3-h collection period also did not differ significantly between the ANS (330 ± 205 μeq) and control (182 ± 116 μeq) groups.

 Effects of reduction of circulating neutrophil numbers by methotrexate. When circulating neutrophils were counted 3 days after the final dose of methotrexate, there was no significant difference from the control levels (Fig. 5).

FIG. 4. Gastric synthesis of 6-keto-prostaglandin F₁α in untreated rats and in rats given indomethacin (20 mg/kg po). Rats receiving indomethacin included control and anti-neutrophil serum-treated (ANS). Each group consisted of 4 rats. ** Significant differences from the untreated group (P < 0.01; Student's t test).
reduction of circulating neutrophil numbers through a nonimmunological means (i.e., treatment with methotrexate) also resulted in a reduction in the susceptibility of circulating neutrophil numbers. Furthermore, the majority of the neutrophils present in smears from rats treated with methotrexate exhibited hypersegmented nuclei. Indomethacin-induced gastric damage in rats challenged 3 days after methotrexate treatment was not significantly different from that in control rats, whereas in the rats challenged 5 days after methotrexate was significantly reduced ($P < 0.01$; Fig. 5).

**Effects of a PAF antagonist.** Treatment with WEB 2086 (3 or 20 mg/kg) before and 75 min after oral administration of indomethacin did not significantly affect the extent of gastric damage. In control rats treated with the vehicle, the mean gastric damage score was 31.8 ± 12.0, whereas in the group treated with WEB 2086, the mean gastric damage scores were 38.8 ± 6.9 (3 mg/kg) and 35 ± 5 (20 mg/kg).

**Effects of a leukotriene D$_4$ antagonist.** Treatment with a specific LTD$_4$ antagonist did not significantly affect the susceptibility to indomethacin induced gastric damage. In the five control rats pretreated with the vehicle, the mean gastric damage score was 50.2 ± 6.1, which was not significantly different from the mean score of 32.8 ± 8.8 observed in the five rats pretreated with MK-571.

**DISCUSSION**

The present results implicate a role for the circulating neutrophil in the pathogenesis of gastric damage induced by nonsteroidal anti-inflammatory drugs. Reduction of circulating neutrophil numbers by treatment with ANS resulted in a significant reduction of the severity of gastric damage induced by either indomethacin or naproxen. Such an effect was not observed when the rats were treated with normal goat serum, suggesting that the effects of the ANS were specifically related to the reduction of circulating neutrophil numbers. Furthermore, reduction of circulating neutrophil numbers through a nonimmunological means (i.e., treatment with methotrexate) also resulted in a reduction in the susceptibility to indomethacin-induced gastric damage. Although methotrexate treatment did not reduce circulating neutrophil numbers to the same extent as the ANS, it is likely that the function of the remaining circulating neutrophils would have been compromised in methotrexate-treated rats. The reduction of NSAID-induced damage in ANS-treated rats was not attributable to-effects on gastric acid secretion nor was it a consequence of reduced cyclooxygenase inhibition, since gastric 6-keto-PGF$_1\alpha$ synthesis was reduced by >95% in both control and ANS-treated rats.

There are several possible mechanisms through which neutrophils might contribute to NSAID-induced gastric ulceration. Neutrophil-derived free radicals have been shown to contribute significantly to the necrosis induced by ischemia-reperfusion in the intestine (15, 16) and to the ulceration induced in the stomach by hemorrhagic shock (10, 20). Free radicals have also been suggested to be involved in the pathogenesis of gastric erosions induced by aspirin (18). As well as directly contributing to tissue necrosis, oxygen-derived free radicals can influence vascular tone by accelerating the inactivation of endothelium-derived relaxing factor (7) and in doing so could alter the resistance of the gastric mucosa to damage. In addition to free radicals, neutrophils can also release proteases that can contribute to gastrointestinal ulceration (17) and to changes in mucosal permeability (21). The studies using Monastral Blue demonstrated that vascular endothelial injury preceded the development of hemorrhagic erosions, and such changes in endothelial integrity were not observed in neutropenic rats. It is therefore possible that the factors responsible for this vascular injury are critically important in the pathogenesis of NSAID-induced ulcers. Whether neutrophil-derived proteases contribute to the changes in vascular integrity and ulceration induced by NSAIDs such as indomethacin requires further study. Neutrophils might also contribute to NSAID-induced gastric damage by producing focal ischemia. Neutrophil aggregates were suggested (28) to contribute to the vascular engorgement and resulting hypoperfusion that follows administration of PAF. Neutrophils have similarly been implicated (21) as contributing factors to the ischemia induced in the stomach by hemorrhagic shock. Such a role of neutrophils would also be consistent with the observations of Kitahora and Guth (12). After exposing the rat gastric mucosa to aspirin, they observed the accumulation of white thrombi within the gastric microcirculation before a reduction in mucosal perfusion and the development of hemorrhagic lesions.

Neutrophils can release various lipid mediators that affect vascular tone and permeability and that might contribute to the ulceration induced by NSAIDs. For example, leukotrienes and PAF have been shown (19, 25, 28) to increase the susceptibility of the gastric mucosa to the damaging actions of ethanol. PAF has also been suggested to play an important role as an activator of neutrophils (23) and to promote adhesion of neutrophils to the endothelium (1). In the indomethacin model of gastric injury, however, these mediators do not appear to play an important role. Changes in gastric LTB$_4$ synthet-
sis, which was monitored as an index of 5-lipoxygenase activity, were not observed after indomethacin administration. Furthermore, pretreatment with either an LTD₄ antagonist or a PAF antagonist did not significantly alter susceptibility to indomethacin-induced gastric damage.

It is conceivable that a balance exists between factors that promote neutrophil activation and factors that inhibit neutrophil activation. Prostaglandins have been shown to inhibit activation of neutrophils (29) and the release of superoxide anions (8). Prostacyclin generation by the vascular endothelium may be particularly important as a tonic inhibitory factor of neutrophil adherence. Through inhibition of prostaglandin synthesis, or perhaps through a mechanism unrelated to effects on prostaglandin synthesis, NSAIDs may tip the balance toward activation of circulating neutrophils. Whether this activation includes accumulation of neutrophils within the gastric microcirculation is not yet known. Further studies are required to establish the mechanism through which neutrophils contribute to NSAID-induced ulceration.

The authors are grateful to Dr. A. Ford-Hutchinson of the Merck-Frosst Centre for Therapeutic Research for the samples of MK-571 and LTB₄, and for the antiserum to LTβ*, and to Dr. H. Heuer of Boehringer-Ingelheim for the sample of WEB 2086. This work was supported by grants from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. J. L. Wallace is the recipient of scholarships from the MRC and AHFMR.

Address for reprint requests: J. L. Wallace, Dept. of Medical Physiology, Univ. of Calgary, Calgary, Alberta T2N 4N1, Canada.

Received 7 September 1989; accepted in final form 8 May 1990.

REFERENCES