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Aggravating Process Induced by Indomethacin on Chronic Gastric Lesion in Rat. Role of Polymorphonuclear Leucocytes

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Abstract

The present study examines the indomethacin-provoked aggravation of chronic ulceration induced by acetic acid in rats. The drug was administered in a single dose, 7 and 14 days after provocation of ulceration. The changes induced by indomethacin in other groups of animals that had been treated for 7 and 14 days with hydroxyurea (which provokes a marked leucopenia) were also studied.

The results obtained demonstrate that indomethacin does not significantly modify the macroscopic index of ulceration nor vascular permeability in the majority of the groups tested. Only in the group that received hydroxyurea for 14 days was there an increase in both parameters. Myeloperoxidase activity was assayed and used as an index of leucocyte infiltration in an attempt to relate the increase in this activity with a gastrolesive effect. Application of acetic acid produced a significant increase in this activity 7 days after induction of chronic injury. Administration of hydroxyurea intraperitoneally was associated with a decrease in the severity of chronic ulceration and neutrophil infiltration into the gastric mucosa. This effect was detectable enzymatically and microscopically. The groups that received indomethacin showed an increase in myeloperoxidase activity, although this increase was only significant in the animals treated with hydroxyurea for 7 and 14 days.

The results suggest that the aggravation provoked by indomethacin is greater when the ulcer curing process is more advanced.

A certain percentage of patients with peptic ulcer lesions will suffer a recurrence of the lesions even after vigorous treatment of the outbreak (Dobrilla et al 1988; Holman et al 1990; Sonnenberg et al 1991). A recurrence of bleeding is expected to occur in about 40% of patients with duodenal ulcers. The mechanisms involved are difficult to establish, although individual responses to drugs, local factors in the digestive tract and environmental influences have been implicated (Piper et al 1978).

There is also a recognized association between the use of non-steroidal anti-inflammatory drugs (NSAIDs) and upper gastrointestinal bleeding (Carson et al 1987; Stodolnik et al 1990). It has been estimated that between 30 and 50% of patients admitted to hospital with upper gastrointestinal bleeding have filled a prescription for NSAID within 30 days, compared with approximately 10% of controls (Carson et al 1987; Langman 1989; Stodolnik et al 1990; Hirschowitz & Lanas 1991). In this way, it is known that the NSAIDs delay the healing of gastric ulcers induced in experimental animals (Szelenyi et al 1982; Wang et al 1989; Bulbena et al 1991; Ogihara & Okabe 1993a). Decrease in the endogenous prostaglandin level (Wang et al 1989; Ogihara & Okabe 1993b) and the gastric mucosal blood flow around ulcer tissue (Hirose et al 1991), and the inhibition of angiogenesis in the granulation tissue (Tarnawski et al 1991) have been postulated to be involved in the underlying mechanism.

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In recent years the role of neutrophils in the pathogenesis of several types of injury to the gastrointestinal tract has become better appreciated. Thus leucocytes have been implicated in the ulceration induced by haemorrhagic shock (Smith et al 1987), ethanol (Kvietys et al 1990; Tepperman & Soper 1990), ischaemia-reperfusion (Hernandez et al 1987) and NSAIDs (Wallace et al 1990; Wallace & Granger 1992).

The present work was designed in an attempt to establish an experimental model allowing the study of the aggravation by indomethacin of chronic gastric lesions induced by acetic acid injection in the rat; we have also evaluated the potential involvement of neutrophils in this aggravating process.

Materials and Methods

Animals and compounds

Male and female Wistar rats (supplied by the animal service, Faculty of Pharmacy, University of Seville), 180–200 g, were fed a normal laboratory diet and tap water. The temperature was maintained at 22–24°C and humidity at 70–75% in a controlled room.

Hydroxyurea, 200 mg kg^{-1} (Sigma Chemical Co, St Louis, MO, USA), a neutropenic agent, was administered intraperitoneally in aqueous solution once daily starting one day before acetic acid application. Indomethacin, 20 mg kg^{-1} (Sigma Chemical Co, St Louis, MO, USA), a cyclo-oxygenase inhibitor, was given orally in aqueous suspension, 3 h before the animals were killed. Control

rats received distilled water orally in a comparable volume (1 mL/100 g).

Induction of chronic gastric injuries

Gastric lesions were induced according to the method described by Okabe & Pfeiffer (1971). Briefly, rats were lightly anaesthetized with diethyl ether and stomachs exposed through medial laparotomy. The antral areas of the stomachs were injected with 0·05 mL 5% acetic acid. This procedure made possible the production of histologically characteristic ulcers, 2–3 days after this injection, with a benign spontaneous evolution (Okabe & Pfeiffer 1971; Navarro et al 1990). Treatment was started 24 h before the procedure and extended for 7 or 14 days after surgery.

Histological evaluation

Animals, 10 for each group, were killed at 7 and 14 days after acetic acid injection. The stomach was exposed and an incision was made in the duodenum. A polyethylene cannula was placed into the stomach via the oesophagus, and the gastric lumen was washed with 10–15 mL saline. To maintain a similar intragastric pressure in all animals, the same amount of formal-buffered fixative solution (10%, pH 7·0) was perfused through a cannula in the gastric cavity. After the solution had emptied through the duodenal incision, the oesophagus and duodenum were ligated. Fifteen minutes later, the stomach was removed and placed in the same fixative solution for 24 h. At the end of this period, the stomach was cut along its greater curvature and the length and width of gastric ulcer were measured determining the ulcer index (mm^2); the central part of the ulcerated tissue was cut in half along the long diameter. The tissue was then embedded in paraffin. Four serial sections 5–7 mm thick were taken, stained with haematoxylin and eosin and examined microscopically (Carl Zeiss II Microscope). For each section the following parameters were noted.

Gastric damage. The stained sections were quantified according to the scale proposed by Bulbena et al (1991) with some modifications: 0 no lesions; 1 minimal lesion or slight oedema of the luminal surface of the mucosa; 2 completely covered lesion with well-defined mucosecretor epithelium, little inflammatory response, significant penetration of granulation tissue with typical arrangement of fibroblast and collagen fibres and a significant degree of vascularization; 3 lesion covered by mucosecretory epithelium, less development of granulation tissue, medium degree of vascularization; 4 epithelization at the margins of the wounded area, necrotic layer covering the lesion, slight granulation tissue development and vascularization; 5 severe erosion of the mucosa with no newly-formed mucosecretory epithelium, widespread necrotic tissue covering the lesion, strong inflammatory response, no revascularization.

Presence of regenerative epithelium. Development of regenerated epithelium was evaluated using the following scale (Hernandez et al 1987): 0 absence; 1 slight presence; 2 mild presence; 3 severe proliferation; 4 completely regenerated.

Infiltration of inflammatory cells. The severity of infiltration was graded from 0 (no infiltration) to 4 (marked inflammatory infiltration).

For each animal, the median value derived from the four slides taken was used for analysis. The assessment was made by a pathologist unacquainted with the experimental findings. When required, photographs were taken using an Olympus OM-UTI camera.

Results are expressed as percentages of the parameters evaluated, 100% being assigned to the maximum point on the scale.

Determination of microvascular permeability

The microvascular permeability (MVP) was evaluated 1 h after the last treatment by measuring the extravasated amount of Evans blue in the mucosa according to Takeuchi et al (1987). Evans blue binds largely to albumin and normally escapes into extravascular tissue in only small quantities, but leaks out in larger amounts if vascular permeability is increased. MVP was determined in new groups of ulcerated animals and treated as previously described for 7 and 14 days after application of acetic acid. In each case, 1 mL 1% Evans blue (w/w) was injected intravenously 30 min before the animals were killed. The stomach was opened and the lesioned tissue and that corresponding to undamaged gastric tissue were isolated, placed in pre-weighed tubes, weighed, and their dye content was extracted in formamide at 65°C for 12 h according to the method described by Ukada et al (1970). Dye concentration was quantified by light absorbance at 620 nm (Perkin-Elmer Lambda 3) and its tissue content (mg Evans blue (mg wet weight tissue)$^{-1}$) was calculated from a standard curve.

Assessment of leucocyte involvement

Neutrophil infiltration in-vivo was assessed by measuring granulocyte specific enzymes such as myeloperoxidase in tissue (Grisham et al 1990; Komatsu et al 1992). Myeloperoxidase activity in this experimental model was measured on days 7 and 14 after ulcer induction in new groups of animals. New control groups were also treated with saline or hydroxyurea, but acetic acid was not injected.

Tissue preparation. Areas of gastric tissue were obtained from lesioned stomachs of animals with induced ulcers and from undamaged stomachs of controls. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry and frozen at −70°C. The assay for myeloperoxidase activity was always performed within two weeks of the experiment. The tissue was thawed, weighed and homogenized in 10 vols 50 mM potassium phosphate buffer (pH 7·4). The homogenate was centrifuged at 20 000 *g*, 20 min, 4°C. The pellet was again homogenized in 10 vols 50 mM potassium phosphate buffer (pH 6·0) containing 0·5% HETAB and 10 mM EDTA. The homogenate was subject to one cycle of freezing/thawing and a brief period of sonication.

Myeloperoxidase assay. Briefly, myeloperoxidase activity was assayed spectrophotometrically using a minor modification of the method which utilizes 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate (Grisham et al 1990; Komatsu et

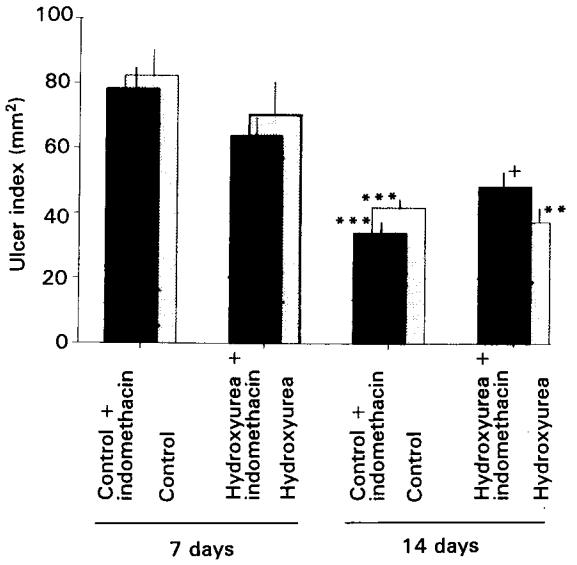

FIG. 1. Ulcer index of control groups, 7 and 14 days after acetic acid application (grey bars). Effect of hydroxyurea (200 mg kg^{-1}, i.p.) at the same periods and modifications induced by indomethacin (20 mg kg^{-1}, p.o.) are also shown (dark bars). $^{**}P<0.01$, $^{***}P<0.001$ compared with respective group at day 7. $+P<0.05$ compared with group without indomethacin.

al 1992). In this method 0·5 mL homogenate is added to a 0·5 mL reaction volume containing 80 mM phosphate-buffered saline (pH 5·4), 0·5% HETAB and 1·6 mM TMB. The mixture was incubated at 37°C for 5 min and the reaction started by the addition of 0·3 mM H_2O_2. Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37°C. The reaction was terminated by the sequential addition of catalase (20 mg mL^{-1}) and 2 mL 0·2 M sodium acetate (pH 3·0). The changes in absorbance at 655 nm were measured with a spectrophotometer (Model Perkin-Elmer Lambda 3). One unit of myeloperoxidase activity was defined as the amount of enzyme present that produced a change in absorbance of 1·0 unit min^{-1} at 37°C in the final reaction volume containing the acetate.

Blood leucocyte counts

New groups of animals were used for blood leucocyte counts. The animals were anaesthetized by inhalation of diethyl ether. The thorax was opened and approximately 2 mL blood was withdrawn by cardiac puncture (using a 21-gauge butterfly needle) and added to vials containing EDTA as an anticoagulant. Blood smears were prepared, and examined microscopically for leucocyte counts by Neubauer camera.

Statistical analysis of data

The data are presented as the mean ± s.e.m., and the statistical analysis employed was Student's *t*-test.

Results

Lesions were macroscopically examined in all animals at 7 and 14 days after acetic acid application. The ulcer index values seen in controls were 82·40 ± 7·10 on day 7 and 41·86 ± 2·63 on day 14 ($P<0.001$), indicating that the ulcers spontaneously diminished in size (Fig. 1). In the control groups that received a single dose of indomethacin in both time periods, a slight, though not significant, decrease was observed in the index. The macroscopic lesions were not modified by hydroxyurea at either 7 or 14 days as compared with the respective controls, but in the animals that received indomethacin 14 days after treatment with the drug, there was a significant increase.

These results were confirmed by microscopic examination (Table 1). The percentage severity of the chronic ulceration as judged by microscopy ranged from 74 ± 12 at day 7 to 60 ± 6 at day 14 ($P<0.05$) in the control groups. The morphology of the microscopic lesion varied from severe erosion of the mucosa without newly-formed mucosecretory epithelium to lesions covered by mucosecretory cells but without a well-structured gastric wall (percentage regenerated epithelium 37 ± 7 at day 7 and 55 ± 7 at day 14 ($P<0.05$). However, in the animals treated with indomethacin a greater proliferation of haemorrhagic erosions could be seen, although one single dose was not enough to change the epithelium regeneration values, nor to increase significantly the neutrophil infiltration (Table 1).

By contrast, lesions completely covered by regenerative tissue, sometimes arranged into columnar structures above granular tissue, with significant degrees of vascularization, were usual in the treated group at day 14 (% ulceration 44 ± 6, $P<0.05$). Regenerative epithelium was also high in this group (70 ± 10, $P<0.05$) and inflammatory infiltration into the gastric lesion was significantly lower ($P<0.01$) at both 7 and 14 days (Table 1). However, in the group treated with indomethacin 14 days after hydroxyurea, there was a worsening of the parameters evaluated.

The increase in the concentration of Evans blue in glandular stomach (MVP) measured 7 and 14 days after acetic

Table 1. Histological evaluation of modification of acetic acid-induced ulcers (7 and 14 days acetic acid).

Modification	Ulcer index (%)		Regenerative epithelium (%)		Inflammatory infiltration (%)	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
None	74 ± 12	60 ± 6+	37 ± 7	55 ± 10	57 ± 7	50 ± 5
Indomethacin	78 ± 4	64 ± 6	35 ± 7	55 ± 10	65 ± 5	62 ± 2
Hydroxyurea	64 ± 8	44 ± 6*	55 ± 9	70 ± 15	32 ± 7**	27 ± 2**
+ indomethacin	62 ± 6	50 ± 8	55 ± 10	65 ± 8+ +	55 ± 10##	57 ± 5##

$^{*}P<0.05$, $^{**}P<0.01$ compared with control; $+P<0.05$, $++P<0.01$ compared with 7-day treatment; $\#\#P<0.01$ compared with corresponding group without indomethacin.

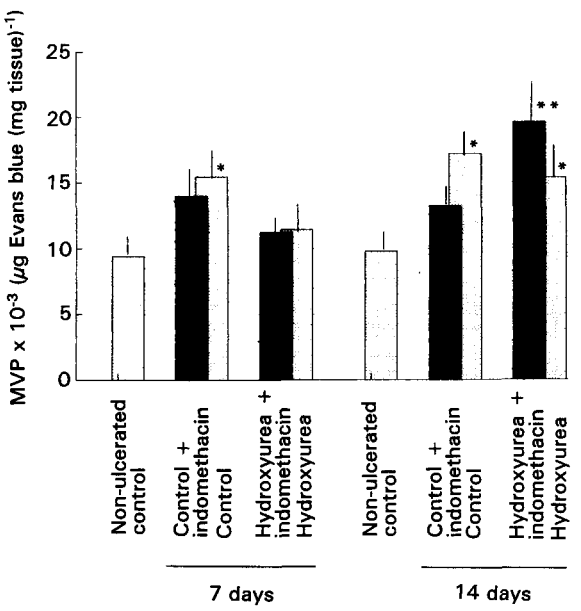

FIG. 2. Microvascular permeability (MVP) of control groups, 7 and 14 days after acetic acid application (grey bars). Effect of hydroxyurea (200 mg kg^{-1}, i.p.) at the same periods and modifications induced by indomethacin (20 mg kg^{-1}, p.o.) (dark bars) compared with the non-ulcerated control are also shown. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with non-ulcerated control.

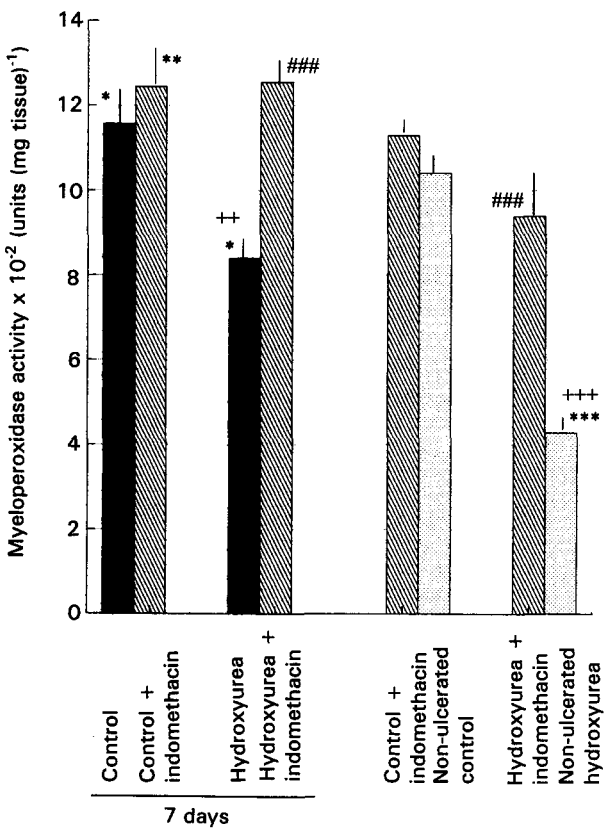

FIG. 3. Myeloperoxidase activity obtained from injured areas in chronic, ulcerated control groups and hydroxyurea treatment (200 mg kg^{-1}, i.p.) after 7 days. Modifications induced by indomethacin (20 mg kg^{-1}, p.o.) are also shown. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ compared with non-ulcerated control. + + $P < 0{\cdot}01$, + + + $P < 0{\cdot}001$ compared with 7-day control group. ###$P < 0{\cdot}001$ compared with respective group without indomethacin.

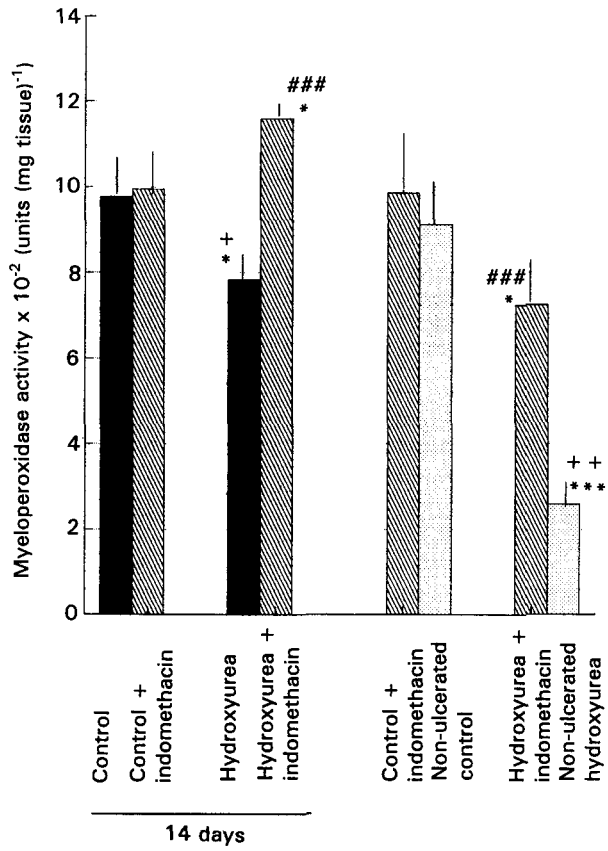

FIG. 4. Myeloperoxidase activity obtained from injured areas in chronic ulcerated control groups and hydroxyurea treatment (200 mg kg^{-1}, i.p.) after 14 days. Modifications induced by indomethacin (20 mg kg^{-1}, p.o.) are also shown. *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$ compared with non-ulcerated control. + $P < 0{\cdot}05$, + + + $P < 0{\cdot}001$ compared with 14-day control group. ###$P < 0{\cdot}001$ compared with respective group without indomethacin.

acid application was statistically significant ($P < 0{\cdot}05$) in both control groups compared with non-ulcerated animals (Fig. 2). These values diminished in the groups treated with hydroxyurea. When the animals were submitted to pretreament with indomethacin, a significant change in the values of MVP was not observed, except in the day-14 group where a significant increase was noted ($P < 0{\cdot}01$ compared with control).

Using myeloperoxidase activity as an index of neutrophil infiltration, we found that the serosal application of acetic acid produced an increase in the lesioned area of the day-7 group (dark column), which was consequently significantly larger than that of the control group ($P < 0{\cdot}05$) (Fig. 3). These results agreed with those found on microscopic examination, which showed a greater infiltration of inflammatory cells in the day-7 group (Table 1). The administration of indomethacin did not appear to modify these results substantially (dashed columns) (Figs 3, 4). In contrast, hydroxyurea treatment provoked a sharp reduction in the myeloperoxidase activity in undamaged animals; the values were significantly lower at both 7 and 14 days than in the control group ($P < 0{\cdot}001$, Figs 3, 4). This fall in myeloperoxidase activity was also observed in areas of chronic lesion, where the values of enzymatic activity were significantly reduced compared with the respective control groups. The

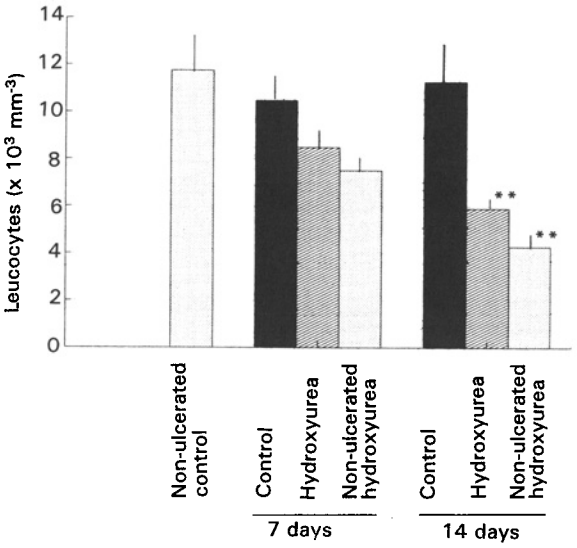

FIG. 5. Blood leucocyte counts in response to hydroxyurea treatment (200 mg kg^{-1}, 7 and 14 days). $^{**}P < 0.01$ compared with non-ulcerated control.

administration of indomethacin in all groups treated with hydroxyurea produced a marked increase ($P < 0.001$) in myeloperoxidase activity.

In addition, hydroxyurea caused a detectable inhibition in the number of circulating leucocytes in blood samples after 14 days of treatment in both acetic acid-treated and undamaged rats (Fig. 5).

Discussion

Gastric ulcers induced by acetic acid in rats are known to resemble human peptic ulcers, both grossly and histologically. The model of Okabe & Pfeiffer (1971) is used widely for studying the effect of drugs on healing rates (Navarro et al 1990; Ogihara & Okabe 1993a). In this experimental model, the chronic gastric lesions were allowed to heal spontaneously for 17 days. This period of time has previously been shown to be sufficient to allow macroscopic healing of the original lesion in 50% of all animals examined (Bulbena et al 1991; Ogihara & Okabe 1993a).

In the present study we have used a potentially damaging agent, indomethacin (20 mg kg^{-1}, p.o.), 7 and 14 days after acetic acid application in an attempt to reactivate chronic gastric lesions. There is a considerable body of evidence suggesting that gastric damage by NSAIDs is causally linked to the ability of these agents to inhibit gastric prostaglandin synthesis (Whittle 1981; Wallace et al 1991). Prostaglandins have potent protective effects in the gastrointestinal tract (Robert 1976) and inhibition of prostaglandin synthesis markedly increases the susceptibility of the gastrointestinal mucosa to injury (Whittle 1983)). In addition, a vascular aetiology for NSAID gastropathy is suggested by both the demonstrable ability of these agents to decrease mucosal blood flow at the ulcer site (Ashley et al 1985; Gana et al 1987; Kitahora & Guth 1987; Miura et al 1991) and by the evidence that vascular endothelial injury in the stomach is an early event after NSAID administration (Rainsford 1983; Tarnawski et al 1990; Wallace et al 1990).

Under our experimental conditions, indomethacin seemed not to contribute to the aggravation of chronic lesions. Furthermore, in general, there was a slight, though insignificant, decrease in the values of the macroscopic ulcer index in animals pre-treated with the NSAID compared with their respective controls, except at day 14, where there was a significant increase in the macroscopic ulcer index. This would seem to be supported by the results of the histological study which did not show differences in the microscopic ulcer index of the animals treated with indomethacin. Only the indomethacin group at 14 days showed aggravation in the microscopic parameter. This could be as a result of the appearance of small, but abundant, haemorrhagic erosions accompanying the treatment. Histological examination indicates that local conditions may favour the aggravation of the original gastric lesions induced by acetic acid in this group (Bulbena et al 1991). Destruction of the lamina propria and proliferation of gastric chief cells in the margins of chronic ulcers was often found closely related to bleeding areas. It seems very likely that the process of repair of the initial lesion that follows the injection of acetic acid results in disorganization of the structures of the mucosa. Under these conditions, proliferation vessels may be unusually located and it is evident that the administration of a single dose of indomethacin contributes to a worsening of the condition.

The general increase in MVP values observed in all groups 14 days after surgery compared with those 7 days after surgery, can be explained by natural angiogenesis. Angiogenesis within granulation tissue is considered one of the most important processes in ulcer healing (Tarnawski et al 1991). Injected Evans blue is distributed exclusively within the vascular lumen and does not extravasate into the interstitial tissue under normal conditions. Fourteen days after acetic acid application, an extensive microvascular network is developed but the gastric wall is not completely restored. This could lead to extravasation of the dye. Tarnawski et al (1991) have demonstrated that indomethacin produces inhibition of angiogenesis in the granulation tissue, and Hirose et al (1991) have shown that this agent decreases the gastric mucosal blood flow around ulcers. This could explain why the MVP values are lower in all groups treated with indomethacin than in their respective controls, with the exception of the animals that received hydroxyurea for 14 days. The fact that in the animals of this latter group indomethacin does not decrease the values of the macroscopic ulcer index nor the MVP values could be due to the fact that the ulcers in these animals are in a more advanced stage of healing so that the anti-inflammatory agent has a different effect. Ogihara & Okabe (1993b) suggest that the mechanism underlying the aggravating action of indomethacin seems to differ in initial, partially healed and healed ulcers. It is possible to explain part of the delayed healing of initial ulcers on the basis of interference in the contraction of the base of the ulcer. However, the aggravation of healed ulcers must involve a mechanism other than the inhibition of the contractile response of the ulcer base because the degree of fibroplasia is too small to interfere with ulcer contraction.

In recent studies, Wallace (1992) has examined the role of neutrophils in the pathogenesis of NSAID gastropathy. NSAIDs cause adherence of neutrophils to the vascular

endothelium, probably through the release of inflammatory mediators that increase the expression of adhesion molecules on the neutrophil (Wallace et al 1990; Asako et al 1992a, b). Adherence of the neutrophil to the endothelium results in reduced perfusion of the mucosa, which predisposes it to injury. Activation of neutrophils as a consequence of stimulation by inflammatory mediators, leukotriene B_4, platelet-activating factor (Rainsford 1987; Wallace 1989; Vaananen et al 1992) and its adherence, results in the release of tissue-damaging factors, such as oxygen-derived free radicals. These factors damage the endothelium and possibly other cells in the mucosa (Vaananen et al 1991).

Our results confirm this fact in the animals that received hydroxyurea, in which leukopenia had been provoked, and subsequently treated with indomethacin. In these groups myeloperoxidase activity was higher than in those groups which were not treated. However, the increase of neutrophil infiltration produced by indomethacin in the animals treated with hydroxyurea for 7 and 14 days was reflected only in an aggravation of the ulcer index in the 14-day group, in which the NSAID also induced an increase of MPV values. These results suggest that indomethacin in this experimental model produces vascular alterations that, in the long term, could delay the healing process.

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